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Impact of transcription and proteolysis in (p)ppGpp-dependent cell cycle control of Caulobacter crescentus

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Impact of transcription and proteolysis in (p)ppGpp-dependent cell cycle control of *Caulobacter crescentus*

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Impact of transcription and proteolysis in (p)ppGpp-dependent cell cycle control of *Caulobacter crescentus*

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Accumulating the hyperphosphorylated guanosine (p)ppGpp is a mechanism used by bacteria to face environmental stresses. Involved in regulation of many essential processes, (p)ppGpp is better known for its role in the stringent response allowing bacteria to face starving conditions. Upon starvation, an arsenal of cellular processes must indeed be modulated in order to adapt and survive. Interestingly, this alarmone controls the cell cycle of the dimorphic *Caulobacter crescentus*. By delaying both G1-S and swarmer-to-stalked cell transitions, (p)ppGpp promotes the spread of a population experiencing starvation. As the RNA Polymerase (RNAP) has been described as the major target of (p)ppGpp in many bacteria, responsible of the transcriptional switch observed upon starvation, we checked whether RNAP might be the causative factor of the cell cycle delay in (p)ppGpp$^+$ cells. To investigate a potential role of transcription in cell cycle control, we worked on (i) a RNAP mutant mimicking the state upon binding of (p)ppGpp and (ii) RNAP mutants unable to bind (p)ppGpp. Interestingly, we found *rpoB*$_{T561A}$ mimics all the effects of RNAP bound by (p)ppGpp, *i.e.* displaying G1 accumulation, higher motility and slower growth, even in a (p)ppGpp^0 background. Unfortunately we did not have enough time to get the (p)ppGpp-blind RNAP mutants. However, the molecular tools to select them have been constructed and we are currently trying to introduce these mutations into *C. crescentus*. As an alternative mechanism to elongate G1/swarmer lifetime, we characterized the effects (p)ppGpp has on the abundance of two cell cycle regulators, CtrA and DnaA. Indeed, the alarmone increases the level of CtrA that silences DNA replication initiation and at the same time clears the DNA replication initiator DnaA from the cell. Since these two proteins are regulated by proteolysis, we checked whether (p)ppGpp could regulate their proteolytic degradation rates and we found that (p)ppGpp strongly inhibits CtrA proteolysis whereas slightly stimulating DnaA degradation. Our work illustrates that the alarmone can regulate important cellular processes such as transcription and proteolysis.

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Love many things, for therein lies the true strength, and whosoever loves much performs much, and can accomplish much, and what is done in love is done well.

–Vincent van Gogh

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Introduction

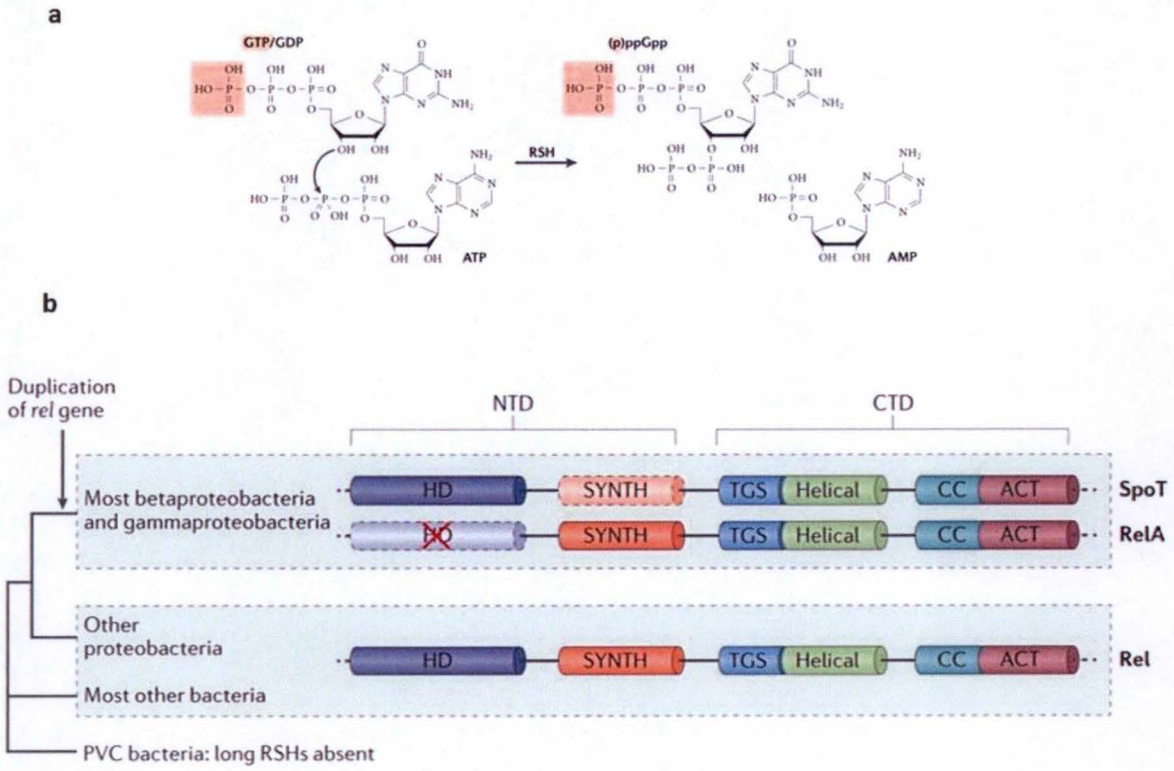

Figure 1 | (p)ppGpp metabolism by RelA, SpoT and Rel. (**a**) The synthesis of guanosine tetraphosphate and guanosine pentaphosphate (collectively referred to as (p)ppGpp) by RelA–SpoT homologue (RSH) family enzymes is shown. The γ-phosphate moiety of GTP and pppGpp are highlighted in red; these moieties are not present in GDP and ppGpp. **(b)** The domains of the long RSHs SpoT, RelA and Rel are shown, along with the distribution of these proteins in bacteria. The coloured boxes representing each domain show their approximate location along the length of the proteins, with dashed borders indicating domains with reduced or absent functional activity. In the case of the SpoT (p)ppGpp synthesis (SYNTH) domain, synthetic activity is weak, whereas hydrolytic activity is completely absent in the RelA (p)ppGpp hydrolysis (HD) domain. The HD and SYNTH domains comprise the amino-terminal domain (NTD), whereas the TGS (ThrRS, GTPase and SpoT), helical, conserved cysteine (CC) and ACT (aspartokinase, chorismate mutase and TyrA) domains together comprise the carboxy-terminal domain (CTD). The phylogenetic tree summarizes the evolutionary relationships among bacteria that contain or lack long RSHs. The arrow indicates the duplication event that led to the emergence of RelA and SpoT from an ancestral Rel protein in the lineage of the Gammaproteobacteria and Betaproteobacteria. Species from the Planctomycetes, Verrucomicrobia and Chlamydiae (PVC) superphylum of bacteria do not encode any long RSHs. In the absence of a reliable root of the bacterial tree of life, it is not known whether long RSHs evolved after the divergence of PVC bacteria, or whether the enzymes were lost in this lineage. P_i, inorganic phosphate; PP_i, pyrophosphate. From *Gerdes et al.*, 2015.

Introduction

I. The guanosine tetra-/penta-phosphate, a general stress response in bacteria

Prokaryotic microorganisms have developed mechanisms to deal with environmental and nutritional stresses, such as the stringent response. Once activated, this response leads to the accumulation of an alarmone, the guanosine tetra-/penta-phosphate referred to as (p)ppGpp. Used by most of bacteria and some plants, these hyperphosphorylated nucleosides are important second messengers that allow adaptation by modulating essential cellular processes such as DNA replication, transcription, translation, ribosome assembly and metabolism (Hauryliuk et al, 2015; Steinchen et al, 2016). The stringent response (SR) was first described in the γ-proteobacteria *Escherichia coli* but has been studied in many other bacteria. In *E. coli*, the SR consists to inhibit the transcription of stable RNA (rRNA), to limit the synthesis of ribosomes, to activate biosynthetic pathways and to arrest growth (Boutte & Crosson, 2013). A wide diversity in signals and conditions have been found to activate (p)ppGpp accumulation. Even if actors of the stringent response are mostly conserved, considerable diversity exists in the starvation cues and regulatory mechanisms that are part of this response (Steinchen et al, 2016; Boutte & Crosson, 2013). Thus the upstream signaling of SR as well as the downstream effects have been shaped to correspond to the particular bacterial lifestyle. Although this complex response was intensively studied, mostly in *E. coli*, many questions remain open, *e.g.* mechanisms leading (p)ppGpp accumulation and direct targets of (p)ppGpp are still poorly characterized.

II. (p)ppGpp synthesis and degradation

The synthesis of guanosine 5'-diphosphate 3'-diphosphate (ppGpp) and guanosine 5'-triphosphate 3'-diphosphate (pppGpp) are synthetized by the transfer of pyrophosphate from ATP to the 3'OH group of GDP and GTP, respectively (**Figure 1a**) (Hauryliuk et al, 2015; Chatterji et al, 2001). There are three major groups of enzymes: Small alarmone synthetases (SAS) and small hydrolases (SAH) and long bifunctional synthetase/hydrolase called RelA/SpoT homologue proteins (RSH) (Potrykus et al, 2008; Steinchen et al, 2016; Boutte & Crosson, 2013). These enzymes are found in all bacteria and can coexist within one species in various combinations. SAS and SAH proteins are found in many bacteria and possibly control (p)ppGpp cytosolic concentrations under a variety of stress conditions (Liu et al. 2015). Yet, these enzymes are less explored and only the SAS protein has been studied in Gram-positive species – *i.e. B. subtilis*. RSH proteins are the most conserved (p)ppGpp synthetases and probably the primary enzymes responsible for (p)ppGpp accumulation during starvation (Boutte & Crosson, 2013; Potrykus et al, 2008).

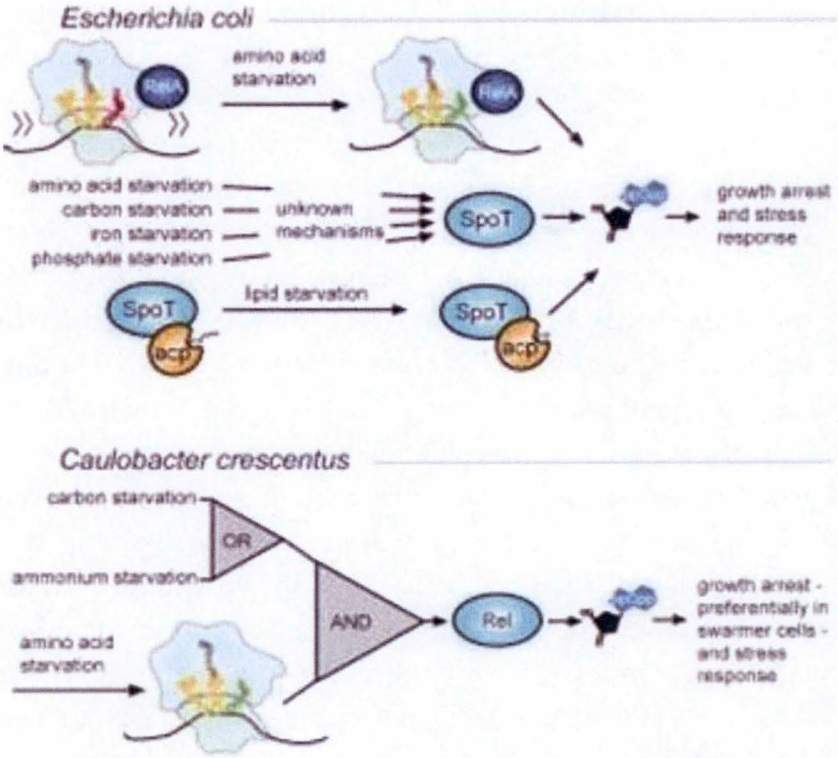

Figure 2 | Stringent response signaling in *Escherichia coli* and *Caulobacter crescentus*. In *Escherichia coli*, several starvation signals can individually activate the SR via either $RelA_{EC}$ or $SpoT_{EC}$. $RelA_{EC}$ is activated at the ribosome when translation is halted due to the entry of an uncharged tRNA in the A site. $SpoT_{EC}$ is activated in lipid starvation through interactions with holo-acyl-carrier protein (Acp) and by several different starvation signals via unknown mechanisms. The SR of *Caulobacter crescentus* has a higher threshold for activation than many other species, requiring both starvation for amino acids AND additional carbon or ammonium starvation signals. The amino acid starvation signal is transmitted through the ribosome, much like $RelA_{EC}$. From *Boutte & Crosson*, 2013.

The architecture of these enzymes are extremely well conserved: the two catalytic domains, hydrolase (HD) and synthetase (SD) are located at their N-terminal extremity, while two "sensing" domains, "ThrS, GTPase and SpoT" (TGS) and "Aspartokinase, Chorismate and TyrA domain" (ACT) are found at their C-terminal extremity (**Figure 1b**) (Steinchen et al, 2016). TGS and ACT play an important role in regulating (p)ppGpp levels by modulating activity of the catalytic domains (Potrykus et al, 2008; Boutte & Crosson, 2013).

In the case of *E. coli*, two RSH enzymes coexist, RelA and SpoT (Potrykus et al, 2008). SpoT harbors both functional synthetase and hydrolase domains whereas HD of RelA is inactivated making it only a synthetase (Steinchen et al, 2016). These two RSH proteins respond to different starving conditions. Under nutrient-replete conditions, RelA enzyme associates with 70S ribosomes. Upon amino acid starvation, uncharged tRNAs accumulate in the ribosomal A-site and stall translation (**Figure 2**) (Hauryliuk et al, 2015) (Boutte & Crosson, 2013). This induces dissociation of RelA from the ribosomes, which stimulates synthesis of (p)ppGpp (Magnusson et al, 2005; Potrykus et al, 2008). At the same time, binding of free uncharged tRNAs to SpoT inhibits its hydrolase activity. As a consequence, (p)ppGpp levels goes up, thereby initiating the stringent response. Most bacteria uses RelA proteins associated with ribosomes to sense amino acids scarcity (Boutte & Crosson, 2013; Hauryliuk et al, 2015). SpoT is sensitive to a plethora of starving conditions (carbon, phosphate, fatty acids, …) (Liu et al. 2015). Except for fatty acids (FA) starvation, no molecular mechanisms have been described for activating SpoT-dependent synthesis of (p)ppGpp in response to the starvation encountered. Upon FA starvation, the holo-acyl carrier protein (holo-Acp), responsible for the growing lipid chains during fatty acids biosynthesis, directly binds to SpoT (**Figure 2**). This binding seems to stimulate SD activity, leading to (p)ppGpp synthesis. The coexistence of two RSH genes in a same bacterium, which sense different starvation, expands the variety of signals to which the species can respond. Furthermore, RelA and SpoT are widely conserved in most γ- and β-proteobacteria, indicating that lipid starvation and amino acid starvation might be regularly encountered in these bacteria (Hauryliuk et al, 2015). Indeed, these starving conditions have been shown to activate (p)ppGpp synthesis in other γ-proteobacteria, In contrast, a single bifunctional RSH (also called Rel) is found in most of the other proteobacteria (Boutte & Crosson, 2013; Potrykus et al, 2008). For example, the α-proteobacteria *C. crescentus* and *S. meliloti* encodes a single Rel responding to only two starvation conditions: carbon and nitrogen (see below).

III. Absence (p)ppGpp leads to pleiotropic effects

Since (p)ppGpp is involved in mostly essential cellular processes, it leads to various changes in cellular physiology including morphological and developmental changes. Indeed, cells lacking (p)ppGpp exhibit severe and pleiotropic defects and cannot properly face to stresses. The alarmone is normally present at basal levels during exponential growth and functions as a major regulator of bacterial growth rate and metabolism. When nutrient are limited, the growth rate decreases and (p)ppGpp redirects cellular resources from ribosome synthesis to stress metabolism (Potrykus et al, 2008). In general (p)ppGpp production is negatively correlated with growth rate. Aberrant cell division and morphology as well as immobility are regularly in bacterial strains unable to produce (p)ppGpp (Liu et at. 2015; Potrykus et al, 2008). In addition, (p)ppGpp has also important roles in the regulation of bacterial biofilm formation, survival and persistence, even in the production of antibiotics in some bacteria (Potrykus et al, 2008; Hauryliuk et al, 2015). It is also important for the virulence of some pathogenic bacteria – *i.e. Pseudomonas aeruginosa*, *Salmonella enterica* or *Mycobacterium tuberculosis*. *Streptomyces antibioticus* and *Streptomyces coelicolor* require (p)ppGpp to synthetize antibiotics, which provide selective advantage over other species in their naturel habitat (Chatterji et al, 2001).

Similarly, *E. coli* produces a secondary metabolite (colicin K) in response to depletion of nutrients, whose production is activated by (p)ppGpp. More than that, the rate of protein synthesis and degradation also depends on cellular growth and may require the (p)ppGpp-dependent control of translation of specific mRNA (Leslie et al, 2015). Finally, (p)ppGpp also inhibits DNA replication, either the initiation step, for instance by inhibiting *dnaA*[1] transcription in *E. coli* or the elongation step, for example by inhibiting the activity of DnaG, the DNA primase (Chatterji et al, 2001; Leslie et al, 2015).

In sum, (p)ppGpp plays a crucial role in both Gram-negative and Gram-positive bacteria non only when bacteria are starved but also in unstressed conditions (Chatterji et al, 2001).

[1] DnaA is the initiator protein used by bacteria to initiate DNA replication by unwind the single origin of replication and loading DNA helicase

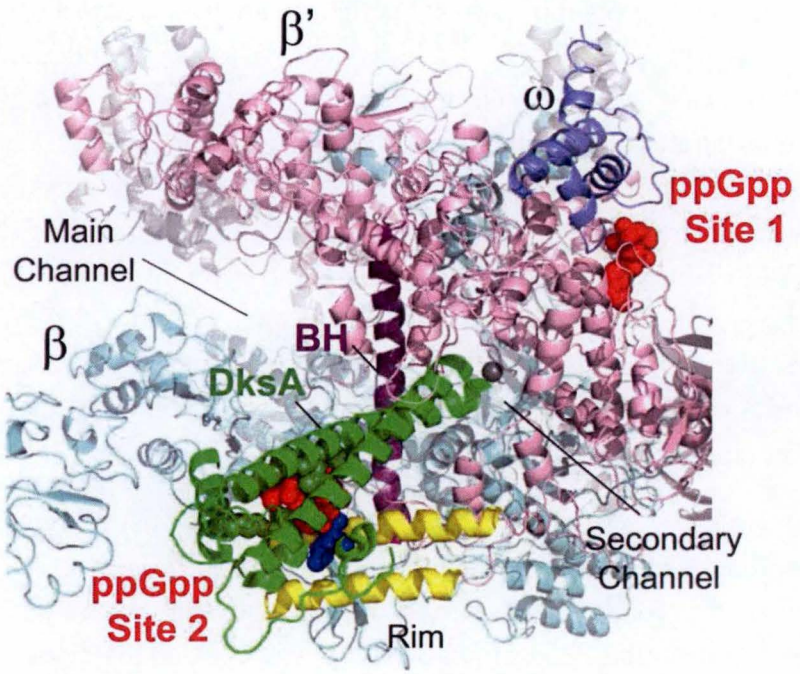

Figure 3 | Model for (p)ppGpp site 2 based on genetic and biochemical data. Model showing proposed location of ppGpp site 2, constructed in Pymol using structures of E. coli RNAP with ppGpp bound at site 1 and DksA .ppGpp, red space-fill; secondary channel rim, yellow; β', pink; β,cyan; ω, slateblue; DksA, green; σ70, gold; β' bridge helix(BH), magenta; active site Mg2+, gray sphere. From *Ross et al.*; 2016.

IV. Cellular targets of (p)ppGpp

To influence bacterial physiology in such a way, the alarmone needs to target key proteins. A few direct targets have been identified and characterized. In general, binding of (p)ppGpp modulates the activity of its target. The best characterized (p)ppGpp targets are (i) the RNA polymerase (RNAP), (ii) enzymes involved in GTP homeostasis and (iii) GTPases associated with ribosomes.

Transcription regulation by (p)ppGpp was first discovered in *E. coli*. Microarrays showed that several hundreds of genes were dependent on (p)ppGpp. In fact, (p)ppGpp showed to regulate transcription both directly and indirectly using a wide range of mechanisms according to the species. A recent study of *E. coli* RNAP showed that (p)ppGpp binds directly into a small pocket between the β' and ω subunits (**Figure 3**) (Lennon et al, 2012; Hauryliuk et al, 2015). Thus in *E. coli*, (p)ppGpp targets RNAP to regulate directly transcription initiation (Boutte & Crosson, 2013). By binding to the interface between the β' and ω subunits, (p)ppGpp can act like an allosteric regulator, which leads to changes of RNAP activity by modifying its conformation (Liu et at. 2015; Magnusson et al, 2005). When (p)ppGpp is bound to RNAP, transcription of genes implicated in proliferation or ribosome protein is downregulated whereas transcription genes implicated in stress and starvation responses are upregulated (Potrykus et al, 2008). In a way, interaction between (p)ppGpp and RNAP may alter promoter selectivity by inducing conformational change in the enzyme. A second (p)ppGpp binding site has been recently discovered. This site 2 requires the atypical transcription factor DksA, originally considered as a (p)ppGpp cofactor since it amplifies the effects of (p)ppGpp and making many promoters more sensitive to (p)ppGpp (Ross et al, 2016). In fact, DksA and (p)ppGpp bind together to the β' subunit of RNAP to destabilize promoter open complexes (Liu et at. 2015; Lennon et al, 2012; Hauryliuk et al, 2015; Potrykus et al, 2008). As a consequence, transcription of stable RNA, ribosomal proteins, fatty acids and flagella is inhibited whereas transcription of amino acid biosynthesis is activated (Potrykus et al, 2008). Interestingly, subunits of RNAP and DksA are very well conserved, indicating that RNAP might be a conserved (p)ppGpp targets (Liu et at. 2015; Ross et al, 2016; Hauryliuk et al, 2015). Site 1 is highly conserved in proteobacteria, but not in more distantly related bacterial phyla where only one of the sites commonly exists (Ross et al, 2016).

However, in most proteobacteria, (p)ppGpp binds both sites 1 and 2 which are both required for responses to nutritional shifts. In *E. coli*, most of the (p)ppGpp^0 phenotypes relies on transcriptional regulation and depends essentially on the (p)ppGpp-binding site 2 (β'/DksA) (Ross et a, 2016).

Effects of (p)ppGpp on transcription initiation can also be indirect since the alarmone also alters the affinity of the housekeeping sigma factor (σ^{70}) to the core RNAP (Magnusson et al, 2005), thereby facilitating the access to alternative σ factor (Potrykus et al, 2008). In this way, (p)ppGpp can favor activation of many promoters and participates to the transcriptional switch. In general σ^{70}-dependent genes involved in cell proliferation and growth – are negatively regulated by (p)ppGpp whereas those implicated in maintenance and stress defense are positively regulated by the alarmone.

Interestingly, Gram-positive bacteria opted for an alternative mechanism to carry out a transcriptional switch upon starvation. In *B. subtilis*, the alarmone inhibits the activity of several enzymes (HprT, GuaB, Gmk) directly involved in GTP synthesis (Kriel et al, 2012; Liu et at. 2015) and, by lowering GTP levels, not only limits transcription initiation of rRNA (GTP being the initiating nucleotide) but also deactivates the transcriptional regulator CodY, thereby allowing transcription of amino acids biosynthetic genes (Liu et al, 2015; Hauryliuk et al, 2015; Boutte & Crosson, 2013).

Finally (p)ppGpp also binds to and modulates activity of GTPases, in particular those involved in ribosome assembly and/or maturation (Corrigan et al, 2016) or in translation efficiency, *e.g.* the translation initiation (IF2) and elongation (EF-Tu, EF-G, …) factors (Rojas et al, 1984; Milon et al, 2006; Mitkevich et al, 2010). It has been shown in *E. coli* that the GTPase CgtA cofractionates with the 50S ribosomal subunit to limit the formation of the 70S ribosomes, and GTP binding to CgtA stimulates the interaction between CgtA and the 50S subunits (Wout et al, 2004; Sato et al, 2005, Gene Cells; Sikora et al, 2006; Jiang et al, 2006; Feng et al, 2014). The role of CgtA in ribosome biogenesis is conserved in *C. crescentus* (Lin et al, 2004; Datta et al, 2004). As CgtA is also directly targeted by (p)ppGpp (Persky et al, 2009), the alarmone could control the translation by this way.

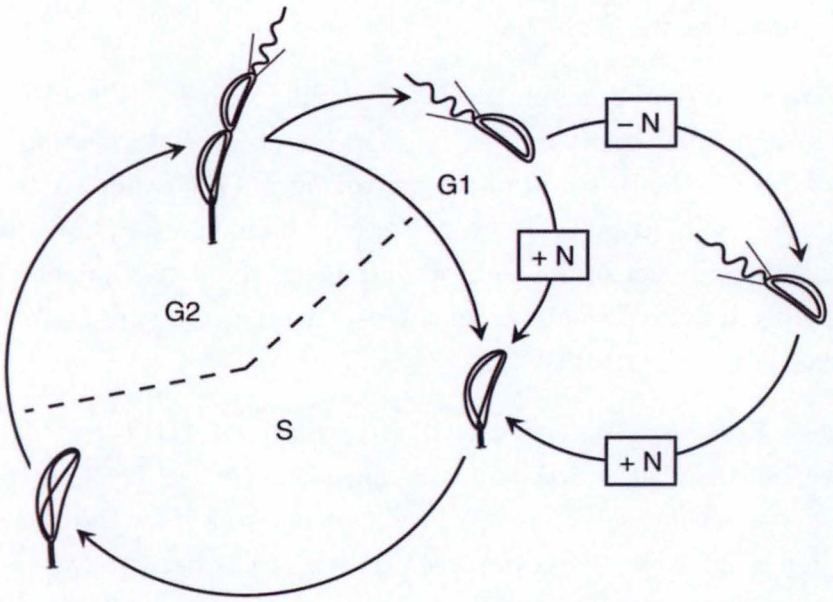

Figure 4 | The *C. crescentus* swarmer cell lifetime is extended upon nitrogen starvation. Asymmetric cell division of *C. crescentus* gives birth to a non-replicative swarmer cell that goes through G1 phase before replicating and a replicative stalked cell that directly enters into S phase. Upon nitrogen starvation (−N), swarmer cells extend their G1 phase. From *Ronneau et al.* 2016

V. *Caulobacter crescentus* is an oligotrophic bacterium dividing asymmetrically

Dividing asymmetrically can be advantageous to adapt to stressing environments. That is what does *Caulobacter crescentus*, an α-proteobacterium that faces a nearly constant nutrient deprivation due to its particular lifestyle. *C. crescentus* is indeed an oligotroph living in highly diluted liquid environments such as pond water. Its asymmetric cell division gives birth to two distinct cell types, a stalked cell and a slightly smaller swarmer cell (**Figure 4**) (Reviewed in Brown et al., 2008, Curtis et al., 2010, Hughes et al., 2012; Ronneau et al, 2016; Boutte & Crosson, 2013). Both cell types have different morphologies and cell cycle potentials. The stalked cell anchored to biotic or abiotic surfaces thanks to the holdfast[2] located at the tip of the stalk, can immediately grow and initiate a new DNA replication cycle at birth whereas the swarmer cell equipped with a polar flagellum, pili and chemotaxis apparatus making it motile, enters first in the pre-replicative G1 phase (Jenal et al, 2009). In nutrient-repleted conditions, a G1/swarmer cell rapidly differentiates into a stalked cell and, concomitantly initiates DNA replication. Once chromosome is replicated, the predivisional cell can divide asymmetrically to generate again a swarmer cell and a staked cell. Interestingly, starving *Caulobacter* cells for nutrients can elongate the G1/swarmer lifetime, a strategy interpreted as a response to starvation since the swarmer cell can escape the poor environment by swimming whereas the stalked cell is sentenced to support starvation (Ronneau et al, 2016). Asymmetric cell division could be a common strategy used by several α-proteobacteria (Hallez et al., 2004) to improve survival. It has been shown that starving a *C. crescentus* population for nitrogen or carbon leads (p)ppGpp accumulation (Chiaverotti et al, 1981; Lesley and Shapiro, 2008; Boutte et al, 2011; Boutte & Crosson, 2013), which ultimately increases the G1/swarmer lifetime (**Figure 4**) (Gorbatyuk and Marczynski, 2004; England et al., 2010; Gonzalez and Collier, 2014).

a. Nitrogen starvation is sensed by the PTS^{Ntr} system to activate (p)ppGpp accumulation

Accumulation of the hyperphosphorylated guanosine (p)ppGpp is associated with nutritional stresses. But how bacterial cells sense specific starvation and induce (p)ppGpp accumulation remains poorly understood. A molecular mechanism has been proposed to sense nitrogen starvation in *C. crescentus* and very likely in other α-proteobacterium. As nitrogen starvation leads to a rapid deprivation of intracellular glutamine, *C. crescentus* measures intracellular pool of glutamine to evaluate nitrogen supply with the help of the nitrogen-related Phosphoenolpyruvate phosphotransferase system referred to as PTS^{Ntr} (Deutscher et al., 2014). The first protein of the PTS^{Ntr}, EI^{Ntr} uses its GAF domain to directly bind glutamine as an allosteric regulator. Once bound to GAF, glutamine inhibits autophosphorylation of EI^{Ntr} with the phosphoryl group coming from PEP.

[2] The holdfast is an exopolysaccharide secreted at the flagellated pole just when stalk biogenesis is initiated

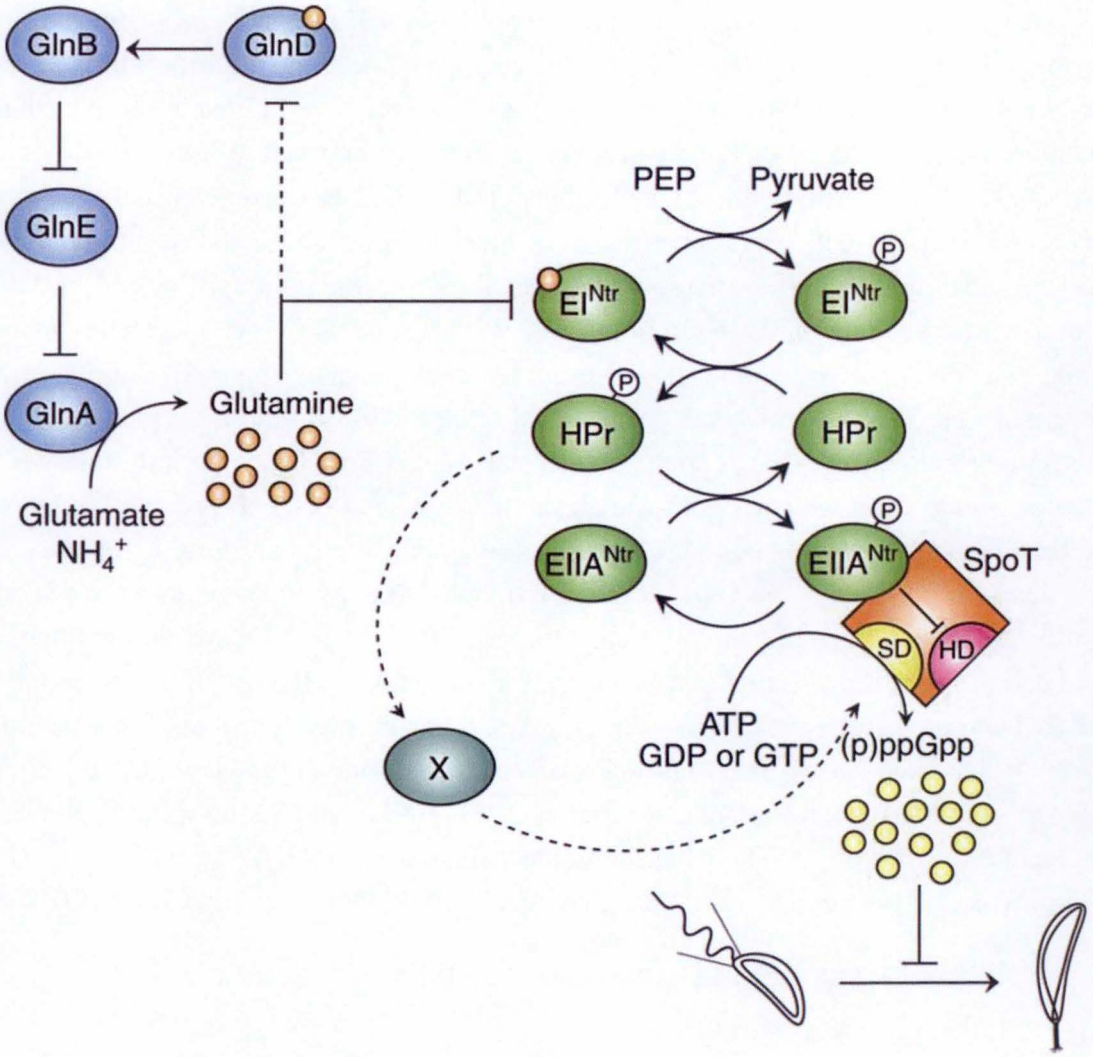

Figure 5 | PTS^{Ntr}-dependent accumulation of (p)ppGpp upon glutamine deprivation. The GlnD/GlnB/GlnE pathway (in blue) regulates glutamine homoeostasis by modulating GlnA (GS) activity. Intracellular glutamine inhibits EI^{Ntr} autophosphorylation, limiting the (p)ppGpp production in +N conditions. Note that the GlnD activity is very likely also inhibited by intracellular glutamine. On nitrogen starvation, intracellular pool of glutamine drops, relieving inhibition of EI^{Ntr} autophosphorylation and thereby increasing HPr and $EIIA^{Ntr}$ phosphorylation levels (in green). Once phosphorylated, $EIIA^{Ntr}$~P interacts with SpoT to inhibit its hydrolase activity (HD), whereas HPr~P regulates indirectly SpoT synthetase activity (SD). This dual control of SpoT by HPr~P and $EIIA^{Ntr}$~P leads to (p)ppGpp accumulation, which in turn delays the G1-to-S and swarmer-to-stalked cell transition. From *Ronneau et al.* 2016

As a result, the downstream PTS^{Ntr} components (HPr and $EIIA^{Ntr}$) are not phosphorylated since these proteins require EI^{Ntr} to be phosphorylated first. In contrast, upon nitrogen starvation EI^{Ntr},HPr and $EIIA^{Ntr}$ will be highly phosphorylated and once phosphorylated, $EIIA^{Ntr}$~P will interact with SpoT, the only (p)ppGpp synthetase/hydrolase bifunctional protein encoded into the genome of *C. crescentus*. By interacting with SpoT, $EIIA^{Ntr}$~P will inhibit (p)ppGpp hydrolase activity, thereby increasing alarmone concentration (Ronneau et al, 2016) (**Figure 5**). The signal "nitrogen starvation" is thus transduced to (p)ppGpp accumulation that leads to cell cycle and development control.

b. (p)ppGpp delays the G1-S transition in *C. crescentus*

One of the most important effect of (p)ppGpp in *C. crescentus* is to delay the G1-S transition by extending the time spent in G1/swarmer phase (Collier et al, 2006; Gonzalez and Collier, 2014). As a consequence, strains overproducing (p)ppGpp – such as $spoT_{D81G}$ which does not have hydrolase activity anymore – has an increased (i) G1 proportion, (ii) motility and (iii) doubling time (Ronneau et al., 2016). Even these phenotypes have been characterized by several labs, the molecular mechanism directly targeted by (p)ppGpp and responsible for the cell cycle delay remains unknown. Two molecular mechanisms might take place, with (p)ppGpp regulating cell cycle regulators abundance and/or transcription by targeting RNAP.

i. (p)ppGpp controls abundance of the cell cycle regulators CtrA and DnaA

Cell cycle progression of *C. crescentus* is allowed by oscillation of at least two global cell cycle regulators: CtrA and DnaA (Reviewed in Brown et al., 2008, Curtis et al., 2010; Collier et al, 2006). CtrA is an essential regulator that controls transcription of many cell cycle genes and inhibits DNA replication by directly binding on the single origin of replication (*Cori*) to silence initiation of DNA replication (**Figure 6**) (Vass et al, 2016; Chien et al, 2007). DnaA is essentially required for DNA replication initiation as it directly binds DnaA boxes – exceptional AT rich regions found in *Caulobacter Cori* – to unwind *Cori* and load DNA helicase (Collier et al, 2006). As DnaA and CtrA act in opposite way, it is not surprising that their respective concentration oscillates in opposite waves, with high CtrA~P/low DnaA concentration in G1/swarmer cells and low CtrA~P/High DnaA levels in S/stalked cells (**Figure 7**) (Taylor et al, 2009). These oscillations come from a combination of transcriptional and post-translational regulations in which proteolytic degradation plays an essential role. Specifically, CtrA activity is regulated during the cell cycle through transcriptional control, directed proteolysis and phosphorylation (Leslie et al, 2015). For instance, CtrA acts as a global transcription factor by directly controlling the expression of about a hundred genes, 26% of total transcripts varying through the cell cycle. CtrA mainly activates early flagellar operons, a chemotaxis operon but also cell division genes or even cell wall biosynthesis genes (Collier et al, 2006; Jenal et al, 2009).

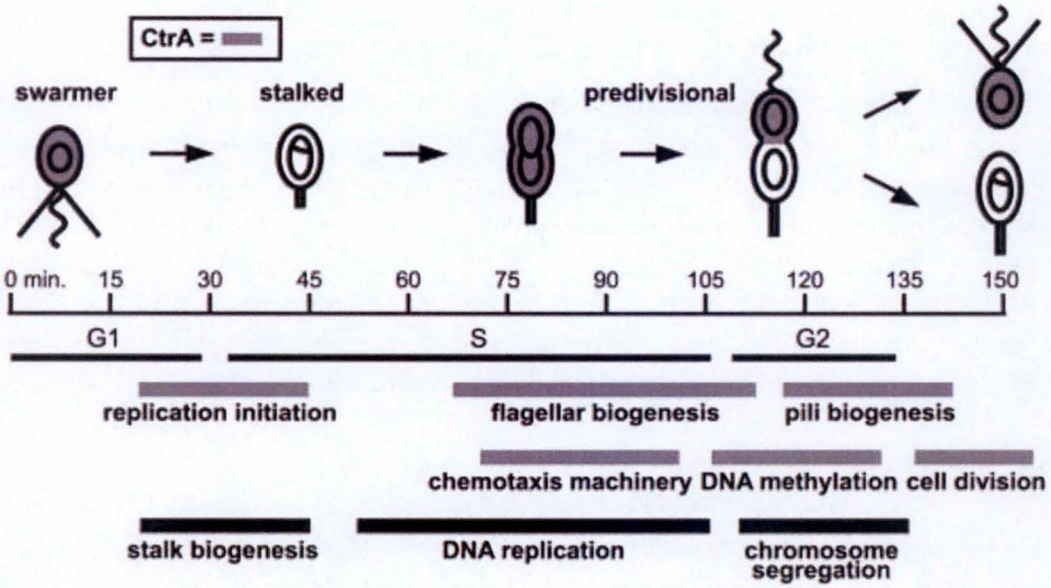

Figure 6 | Temporal coordination of *Caulobacter* cell cycle events. The swarmer cell has a nonreplicating chromosome and a polar flagellum and pili. At the swarmer-to-stalked cell transition, the pili and the flagellum are lost, and a stalk is formed at that same pole, coincident with the initiation of DNA replication. Construction of a new flagellum at the pole opposite the stalk occurs during S phase. The cell then divides asymmetrically, yielding two distinct daughter cells: a stalked cell that initiates a new round of DNA replication and a smaller swarmer cell that cannot replicate its chromosome until after it differentiates into a stalked cell. Timing of several key cell cycle-regulated events is indicated by the black and gray bars below. CtrA is present in cell types shaded gray and controls genes or events involved in the cell cycle processes with gray timing bars. From *Laub MT et al.* 2002.

Several mechanisms have been reported to modulate DnaA activity in many bacteria and ensure the correct timing of DNA replication. RIDA (regulatory inhibition of DnaA) is the major mechanism, found in *E. coli* and other proteobacteria, which relies on ATP binding and hydrolysis (Leslie et al, 2015). Bound to ATP, DnaA is a favored active conformation promoting duplex unwiding. After initiation, ATP hydrolysis is stimulated by HdaA protein and inactivates DnaA (Leslie et al, 2015; Wargachuk et al, 2015). Thereby this mechanism prevents re-initiation of DNA replication. Under scarcity of nutrients, regulation of DnaA activity is composed of decreased translation and constitutive degradation. Likewise, the steady-state levels of both proteins are regulated upon stress (Jenal et al, 2009). For instance, it has been shown that two different ATP-dependent proteases degrade DnaA pool upon stress, Lon after a proteotoxic stress and ClpAP during stationary phase. In both cases, clearance of DnaA is interpreted as a safe mechanism to avoid DNA replication during stress. CtrA is degraded by the ATP-dependent ClpXP, at the G1-S transition as well in the stalked compartment of the late predivisional cells (Liu et al, 2016; Marczynski & Shapiro, 2002; Chien et al, 2007; Collier et al, 2006; Vass et al, 2016). To degrade CtrA at the right time in the right cell, *C. crescentus* uses hierarchical adaptors that are very well conserved in α-proteobacteria (**Figure 8**) (Joshi et al, 2015). First, ClpXP needs to be recruited to the differentiating pole by the unphosphorylated form of CpdR to degrade CtrA. Given that CpdR is phosphorylated by the same phosphorelay than CtrA, its phosphorylation prevents the proteolytic degradation of active CtrA~P. On the contrary, the inactivation of CtrA by dephosphorylation leads to its concomitant degradation since CpdR is dephosphorylated at the same time (Marczynski & Shapiro, 2002). Finally, the adaptor PopA in complex with c-di-GMP helps in delivering CtrA to the primed CpdR::ClpXP protease (Vass et al, 2016; Taylor et al, 2009; Chien et al, 2007). Thus CtrA is not anymore degraded in the absence of *rcdA* or *cpdR* (Joshi et al, 2015).

Several studies highlighted a role of (p)ppGpp in regulating concentration of CtrA and DnaA (Lesley and Shapiro, 2008; Gorbatyuk and Marczynski, 2004; England et al., 2010; Gonzalez and Collier, 2014). The alarmone has been shown to increase CtrA levels and strongly reduce DnaA concentration (Taylor et al, 2009). Even these effects make sense with the effect of (p)ppGpp on cell cycle progression, *i.e.* delay of the G1-S transition, the molecular mechanism behind these effects remains to be characterized.

Nevertheless, post-transcriptional modifications were highlighted to reduce DnaA levels during nutrient limitation and entry in stationary phase. The mechanism used to limit DnaA includes a reduction of DnaA translation rate together with a constant proteolytic degradation rate by the protease Lon (Marczynski & Shapiro, 2002; Vass et al, 2016), this latter having been shown before to degrade DnaA after a proteotoxic stress (Jonas et al., 2013). The regulation of DnaA translation is mediated by a 5'UTR in the *dnaA* transcript (Leslie et al. 2015). Yet, this mechanism is far from being well understood (see *Perspectives)*.

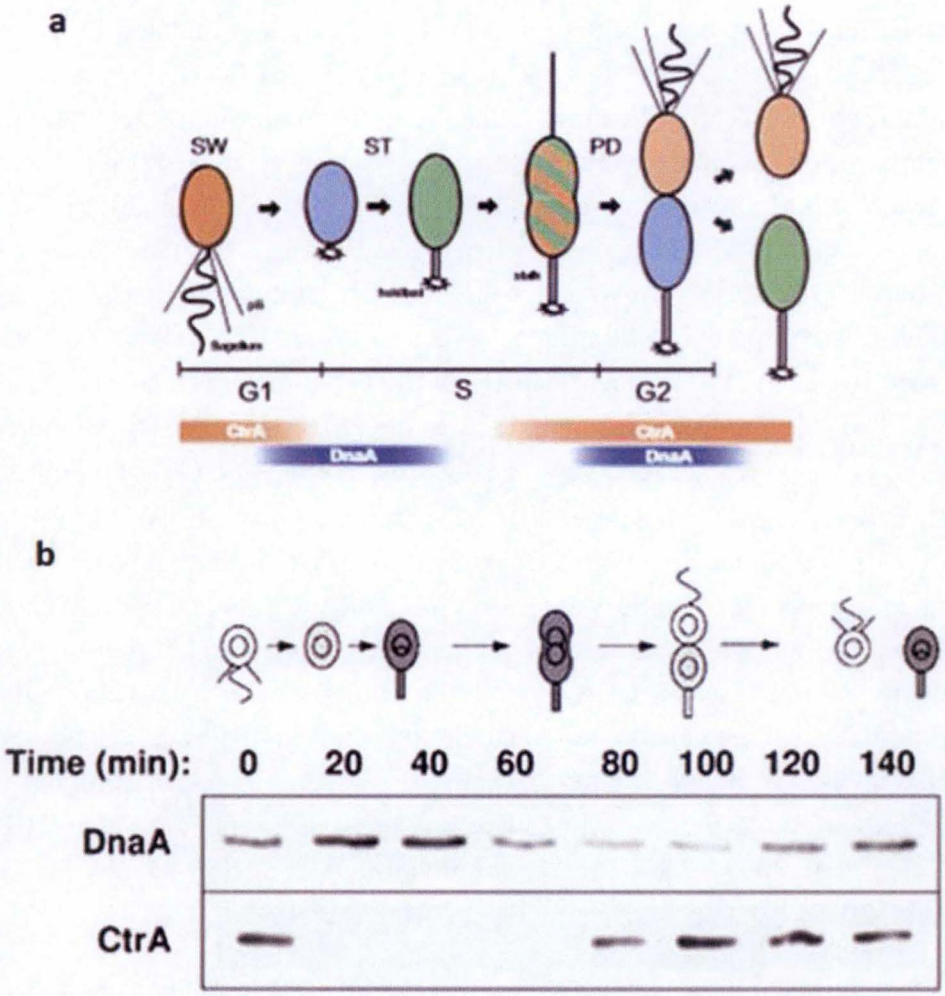

Figure 7 | Oscillation of the global regulators CtrA and DnaA throughout the cell cycle of *C. crescentus*. (**a**) Schematic of the *C. crescentus* cell cycle with the spatial and temporal distribution of CtrA (red), DnaA (blue). The swarmer (SW), stalked (ST) and predivisional (PD) cell types are indicted and polar appendices are labeled. The temporal distribution of two master regulators during the cell cycle is indicated a colored bars. Picture is from *Jenal, 2009* (**b**) Immunoblots of cells extracts from a synchronized NA1000 culture using DnaA and CtrA antibodies at the indicated time of the cell cycle showing the cell cycle oscillation of these three proteins. Picture is from *Shapiro L. et al.;* 2006.

ii. (p)ppGpp might control transcription by binding to RNAP in C. crescentus

Transcription of several hundred of genes is impacted by (p)ppGpp in *E. coli* (Traxler et al., 2008) and *C. crescentus* (Boutte et al., 2011). Knowing that both (p) (p)ppGpp-binding sequences identified on *E. coli* RNAP is highly conserved in *C. crescentus*, the transcriptional regulation induced by (p)ppGpp might be due to the direct binding of the alarmone on the RNAP. However, no studies are available to challenge this hypothesis.

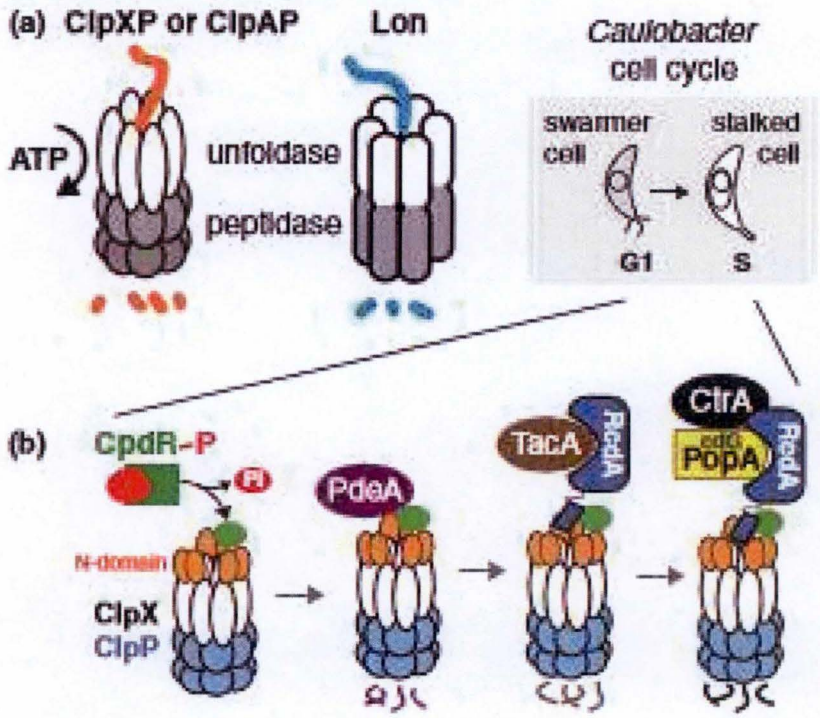

Figure 8 | Protein degradation by energy dependent proteases can be shaped by hierarchical adaptors. (a) The ClP family of proteasesare composed of unfoldases (ClpX or ClpA) paired with the ClpP peptidase. The Lon protease is a single polypeptide with these activities contained in different domains. **(b)** The G1-S transition in Caulobacter is accompanied by morphological changes from a motile swarmer cell to a sessile stalked cell. At this transition, the dephosphorylation of CpdR initiates the assembly of an adaptor hierarchy that results in staged degradation of substrates. From *Vass et al.*; 2016.

Objectives

Objectives

In *C. crescentus*, the (p)ppGpp accumulated upon starvation delays the entry into the cell cycle by extending the G1/swarmer lifetime. But the mechanism targeted by the alarmone to mediate this effect is unknown. Based on the data available in the literature, we made two non-exclusive hypotheses. The delay of the G1-S transition might be due to (i) the combined accumulation of the DNA replication inhibitor CtrA and the disappearance of the DNA replication initiator DnaA and/or (ii) the transcriptional regulation mediated by (p)ppGpp-bound RNAP.

In a first part, we characterized the abundance of DnaA and CtrA depending on (p)ppGpp levels. More precisely, we checked whether the proteolytic degradation of these two proteins could be regulated by (p)ppGpp. In a second part, we tried to determine of transcription regulated by (p)ppGpp might be responsible for the cell cycle control. For that we sought to generate and characterize RNAP mutants, one in *rpoB* (encoding the ß subunit) mimicking the (p)ppGpp-bound state of RNAP and the other in *rpoC* (encoding the ß' subunit) annihilating (p)ppGpp binding.

Results

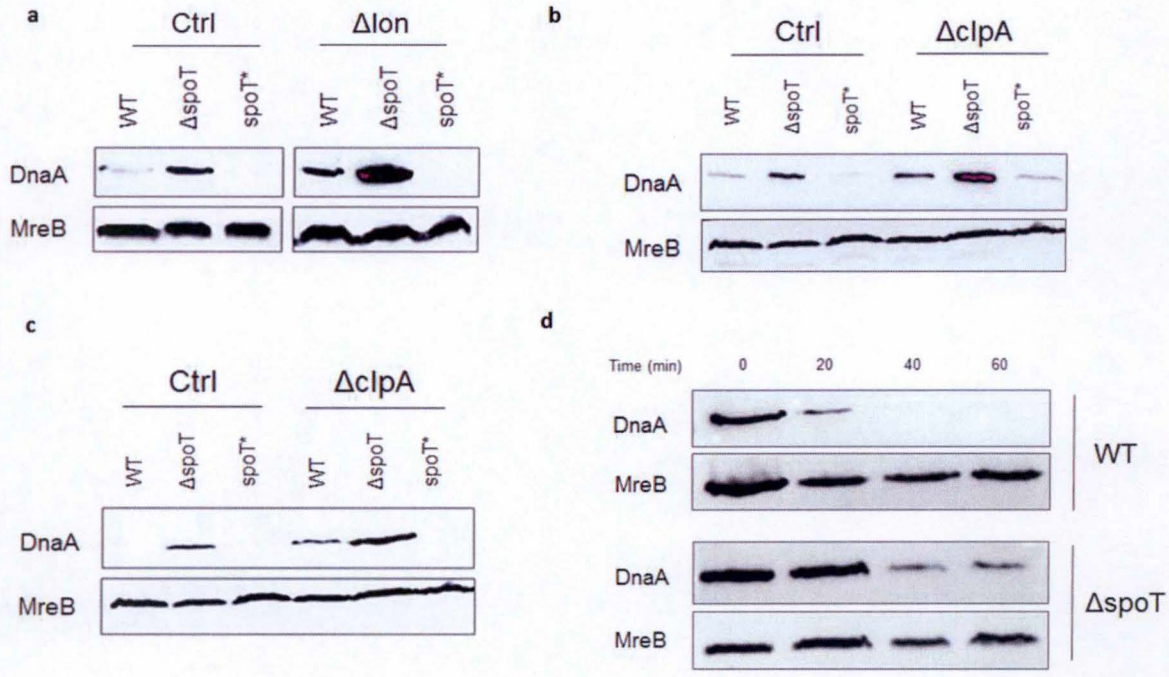

Figure 1 | Abundance and degradation rate of DnaA. Proteins were extracted (**a**) from WT, *ΔspoT, spoT*$_{D81G}$, *Δlon*, *Δlon ΔspoT* and *Δlon spoT*$_{D81G}$ cells cultivated in complex PYE medium up to OD_{660} between 0.4 - 0.6 (mid exponential phase); (**b**) from WT, *ΔspoT, spoT*$_{D81G}$, *ΔclpA, ΔclpA ΔspoT* and *ΔclpA spoT*$_{D81G}$ cells cultivated in complex PYE medium up to OD_{660} between 0.4 - 0.6 (mid exponential phase); (**c**) from WT, *ΔspoT, spoT*$_{D81G}$, *ΔclpA WT, ΔclpA ΔspoT, ΔclpA spoT*$_{D81G}$ cells cultivated in complex PYE medium up to OD_{660} between 1.0 - 1.2 (early stationary phase); (**d**) every 20 minutes after the addition of chloramphenicol (30µg/ml) from WT and *ΔspoT* cells cultivated in complex PYE medium up to OD_{660} between 0.4 - 0.6. All the proteins extracts were subjected to electrophoresis, transferred onto nitrocellulose membranes before being immunoblotted with the following antibodies: α-DnaA (1:2,000), α-MreB (1:5,000) and α-rabbit (secondary antibodies, 1:5,000).

Results

I. (p)ppGpp controls abundance of DnaA and CtrA

To check if (p)ppGpp had an effect on abundance of DnaA and CtrA, we compared levels of both proteins from *C. crescentus* strains producing more [(p)ppGpp$^+$, NA1000 *spoT*$_{D81G}$] or no (p)ppGpp [(p)ppGpp^0, NA1000 Δ*spoT*] by western blots using polyclonal antibodies directed against DnaA or CtrA. As expected, DnaA was more abundant in Δ*spoT* than in wild-type (WT) while it was almost undetectable in *spoT*$_{D81G}$ (**Figures 1a-b**). We also confirmed that (p)ppGpp increases CtrA level since CtrA was much more abundant in *spoT*$_{D81G}$ than in wild-type. In contrast, absence of (p)ppGpp (Δ*spoT*) slightly decreases CtrA level (**Figures 1a-b and 2a**). In conclusion we confirmed (p)ppGpp strongly impacts levels two central regulators of G1-S transition, by enhancing CtrA abundance while stimulating DnaA disappearance. Since both proteins were described to be subjected to proteolysis, making them fluctuating throughout the cell cycle, we tested whether the (p)ppGpp-dependent fluctuation of DnaA and CtrA came from regulation of ATP-dependent proteases.

a. Control of DnaA abundance by (p)ppGpp

DnaA has been described to be degraded by two different ATP-dependent proteases, Lon and ClpAP. To test if these proteases are controlled by (p)ppGpp, we constructed knock-out mutants of *lon* and *clpA* (Δ*lon* and Δ*clpA*) in the following backgrounds: WT *spoT*, *spoT*$_{D81G}$ [(p)ppGpp$^+$] and Δ*spoT* [(p)ppGpp^0]. Indeed if (p)ppGpp stimulates proteolysis of DnaA, deleting the gene coding for the protease responsible for DnaA clearance might suppress the effect we observed in (p)ppGpp$^+$ and (p)ppGpp^0 strains. As expected, the deletion of *clpA* or *lon* led to a higher DnaA levels in strains producing WT level of (p)ppGpp (compare WT Δ*lon* and WT Δ*clpA* to WT Ctrl in **figure 1a-b**). A similar effect was observed in Δ*spoT* background since the DnaA level was higher when *lon* or *clpA* is absent (compare Δ*spoT* Δ*lon* and Δ*spoT* Δ*clpA* to Δ*spoT* Ctrl in **figure 1a-b**). However, deletion of *lon* or *clpA* did not prevent DnaA clearance from (p)ppGpp$^+$ strain. Thus (p)ppGpp still modulates DnaA abundance even in the absence of Lon or ClpA. Although both proteases are able to degrade DnaA *in vitro*, ClpAP has been shown to be activated in stationary phase to clear DnaA. Therefore, we evaluated the DnaA levels in the same strains cultivated up to stationary phase. As illustrated in **figure 1c**, deletion of *clpA* still led to increase of DnaA levels in WT and Δ*spoT* backgrounds (compare WT to Ctrl) but did not avoid DnaA clearance in a (p)ppGpp$^+$.

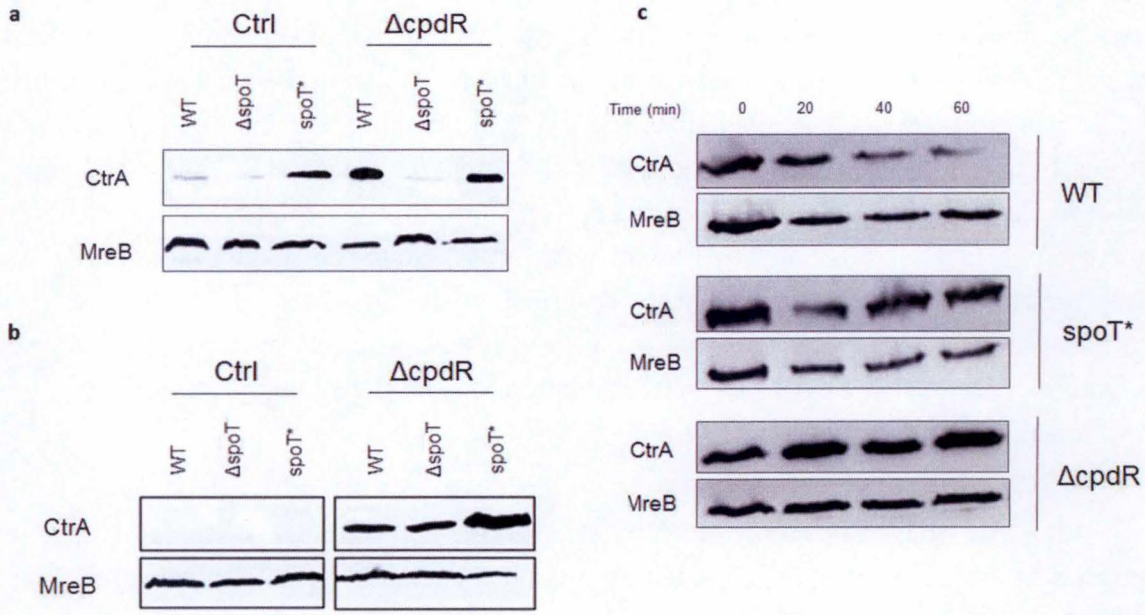

Figure 2 | Abundance and degradation rate of CtrA. Proteins were extracted (**a**) from WT, *ΔspoT, spoT*$_{D81G}$, *ΔcpdR*, *ΔcpdR ΔspoT* and *ΔcpdR spoT*$_{D81G}$ cells cultivated in complex PYE medium up to OD_{660} between 0.4 - 0.6 (mid exponential phase); (**b**) from WT, *ΔspoT, spoT*$_{D81G}$, *ΔcpdR*, *ΔcpdR ΔspoT* and *ΔcpdR spoT*$_{D81G}$ cells cultivated in complex PYE medium up to OD_{660} between 1.0 - 1.2 (early stationary phase); (**c**) every 20 minutes after the addition of chloramphenicol (30µg/ml) from WT, *spoT*$_{D81G}$ and *ΔcpdR* cells cultivated in complex PYE medium up to OD_{660} between 0.4 - 0.6. All the proteins extracts were subjected to electrophoresis, transferred onto nitrocellulose membranes before being immunoblotted with the following antibodies: α-DnaA (1:2,000), α-MreB (1:5,000) and α-rabbit (secondary antibodies, 1:5,000).

Collectively these results indicate that (p)ppGpp does not regulate DnaA levels by controlling the activity of Lon or ClpA protease. To further characterize the potential effect of (p)ppGpp on DnaA proteolysis, we measured the degradation rate of DnaA by determining DnaA levels in wild-type and (p)ppGpp^0 strains grown in culture media supplemented with chloramphenicol. The addition of chloramphenicol avoids neo-synthesis of proteins by irreversibly binding to the 50S subunit of the bacterial ribosome, thereby setting DnaA levels at the beginning of the experiment. We then measured DnaA levels over time (**Figure 1d**) and found degradation of DnaA is slightly slower in a strain lacking (p)ppGpp compared to the wild-type strain ($t_{1/2}$ ~ 40 min). However, this slight effect cannot explain the strong accumulation of DnaA in Δ*spoT* cells.

b. Control of CtrA abundance by (p)ppGpp

The proteolysis of CtrA depends on the ClpXP protease and requires the adaptor CpdR. As for DnaA, we checked whether (p)ppGpp might regulate CtrA abundance by controlling ClpXP activity. To this end, we constructed mutants (*ΔcpdR* and *ΔrcdA*, known to prevent ClpXP-dependent proteolysis of CtrA) in the following genetic backgrounds: wild-type, (p)ppGpp^0 and (p)ppGpp$^+$. Although deletion of *cpdR* led to CtrA accumulation in *spoT* wild-type backgrounds (WT), it did not substantially increase CtrA levels in *spoT*$_{D81G}$ [(p)ppGpp$^+$] backgrounds. These results suggest that ClpXP-dependent proteolysis of CtrA is turned off in (p)ppGpp$^+$ cells (**Figure 2a**). This conclusion is further reinforced by the fact that the degradation rate of CtrA is strongly inhibited by (p)ppGpp. Indeed, CtrA is almost completely stabilized in *spoT*$_{D81G}$ cells after addition of chloramphenicol to the growth medium while it is rapidly cleared from wild-type cells grown in the same conditions (**Figure 2c**).

Interestingly, the deletion of *cpdR* did not prevent CtrA disappearance in a (p)ppGpp^0 background (**Figure 2a**), suggesting that even if (p)ppGpp might stabilize CtrA by inhibiting its proteolytic degradation, it could also promote CtrA abundance by another way. This second mechanism is also visible in *spoT*$_{D81G}$ Δ*cpdR* cells reaching stationary phase since CtrA levels in these cells are higher than in Δ*spoT* Δ*cpdR* or Δ*cpdR* cells (**Figure 2b**).

In conclusion, we showed that (p)ppGpp (i) decreases DnaA levels without enhancing its proteolysis by Lon or ClpAP, (ii) stabilizes CtrA by inhibiting its ClpXP-dependent proteolytic degradation and (iii) increases CtrA levels by second yet unidentified mechanism.

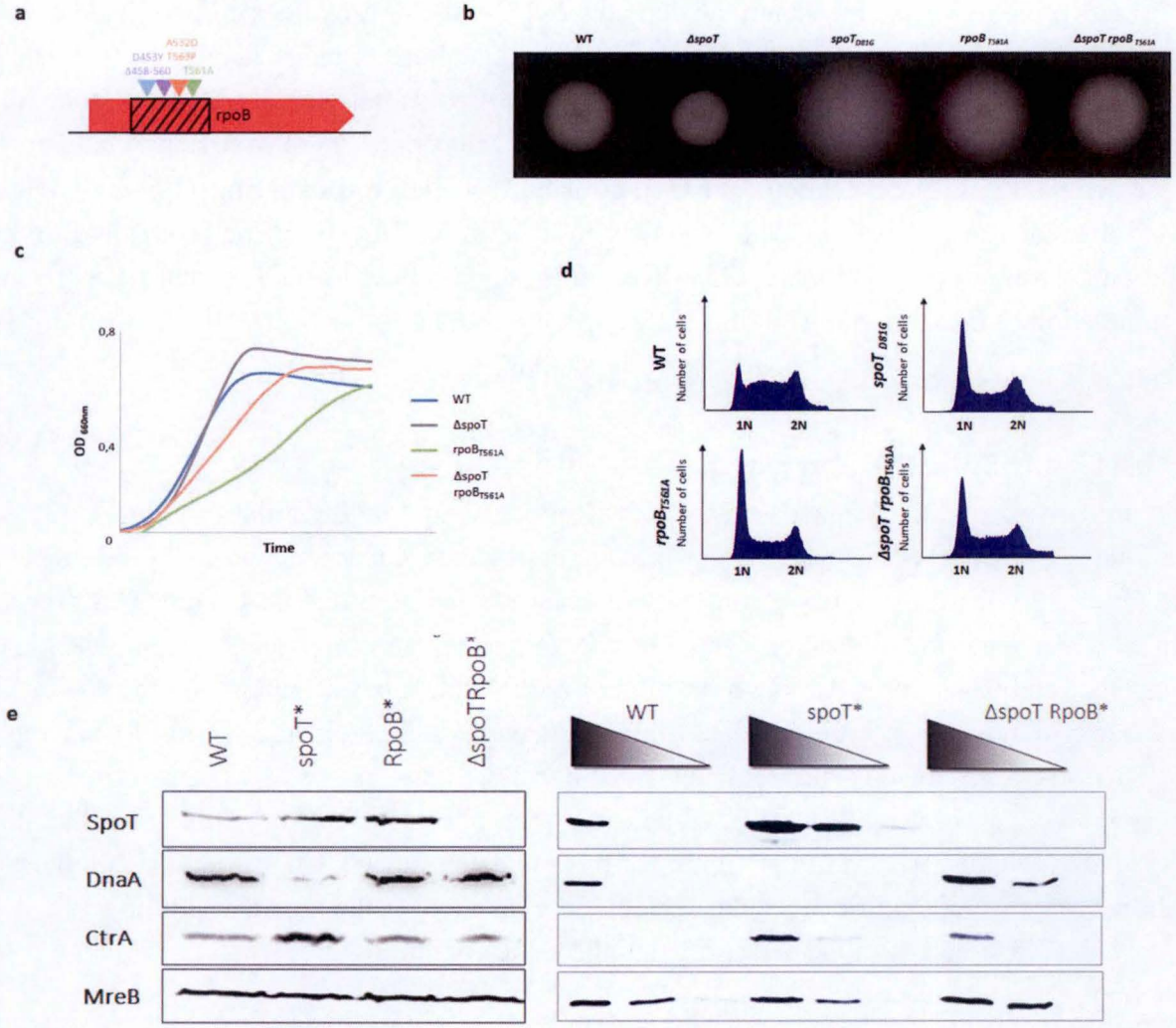

Figure 3 | Mutations in *rpoB* mimic the modification induced upon (p)ppGpp binding to RNAP and show 'stringent response' phenotypes. (**a**) Examples of *rpoB* mutations isolated in different organisms to express stringent response. Cited mutations have been found in *Streptomyces coelicolo*r (blue; Xu et al), *Sinorhizobium meliloti* (purple; Wells et al.), *Escherichia coli* (orange; Harinarayanan et al.) and *Caulobacter crescentus* (green). Growth (**b**), DNA content (**c**) and motility (**d**) of wild-type (WT), *Δspot*, $spoT_{D81G}$, $rpoB_{T651A}$ and *ΔspoT* $rpoB_{T561A}$ grown in complex media (PYE). (**e**) Immunoblots on proteins extracted from WT, $spoT_{D81G}$, $rpoB_{T561A}$ and *ΔspoT* $rpoB_{T561A}$ cells cultivated in complex PYE medium up to OD_{660} between 0.4 - 0.6 (mid exponential phase). The right part corresponds to gradient of each cellular extract (1:1, 1:3 and 1:9). Antibodies used are α-CtrA (1:2000), α-DnaA (1:2000), α-SpoT (1:5000) and α-MreB (1:5000) as control; all the proteins extracts were subjected to electrophoresis, transferred onto nitrocellulose membranes before being immunoblotted with the following antibodies: α-rabbit (1:5000).

II. *rpoB* mutant that mimics (p)ppGpp-bound state of RNAP

We performed a genetic screen based on rifampicin resistance with the aim of highlighting the role of RNAP upon (p)ppGpp accumulation. Indeed, it has been shown that mutations appearing spontaneously in *rpoB* and *rpoC* genes (coding respectively for β and β' subunits of RNAP) to confer rifampicin resistance can also mimic the (p)ppGpp-bound state of RNAP Wells et al, 2003; Xu et al, 2002; Brandis et al, 2012; Tedin et al, 1992). We therefore looked for such mutations in *C. crescentus, i.e* conferring resistance to rifampicin (Rif^R) and mimicking RNAP once bound to (p)ppGpp.

As a first step, a plasmid conferring kanamycin resistance was inserted close to the *rpoBC* locus in a WT strain of *C. crescentus*. Then we selected ~30 spontaneous mutants of this strain that were able to grow on PYE + Rif plates. After checking their DNA content, we focused on mutants showing G1 accumulation – *i.e.* 11 mutant strains. By mimicking (p)ppGpp-bound state of RNAP, mutations should indeed elongate G1 lifetime. ΦCR30 bacteriophage lysates were then prepared for each of the 11 candidates and transduced in both wild-type and (p)ppGpp^0 (Δ*spot*) strains. Transductants were selected on PYE + Kan plates and Rifampicin resistance (Rif^R) as well as G1 accumulation were subsequently tested. Three of the 11 candidates Rif^R / Kan^R showed highest G1 accumulation and two of them harbored the same mutation in *rpoB*, an alanine instead of a threonine at position 561 of the ß subunit ($rpoB_{T561A}$). This mutation is located in a region of *rpoB* where several mutations were found to mimic the (p)ppGpp-bound state of RNAP in other bacterial species (**Figure 3a**) (Xu et al, 2002; Wells et al, 2003, Tedin et al, 1992, Brandis et al, 2012). This mutant strain not only accumulated G1/swarmer cells but also increased doubling time and motility, even in the absence of (p)ppGpp (**Figure 3b-d**). Interestingly, these phenotypes were already observed in a strain overproducing (p)ppGpp [$spoT_{D81G}$, (p)ppGpp$^+$]. Thus the isolated mutation $rpoB_{T561A}$ seems to mimic modification induced upon (p)ppGpp binding to RNAP, even in a Δ*spoT* background. However, in contrast to $spoT_{D81G}$, $rpoB_{T561A}$ did not change CtrA and DnaA levels.

Altogether, these results suggest that the (p)ppGpp-dependent control of cell cycle relies on transcriptional regulation in contrast whereas the control of CtrA and DnaA levels does not depend on transcription. Alternatively, $rpoB_{T561A}$ could inhibit G1-S transition independently of mimicking (p)ppGpp effects, *e.g.* by inhibiting transcription of genes strictly required to enter the cell cycle.

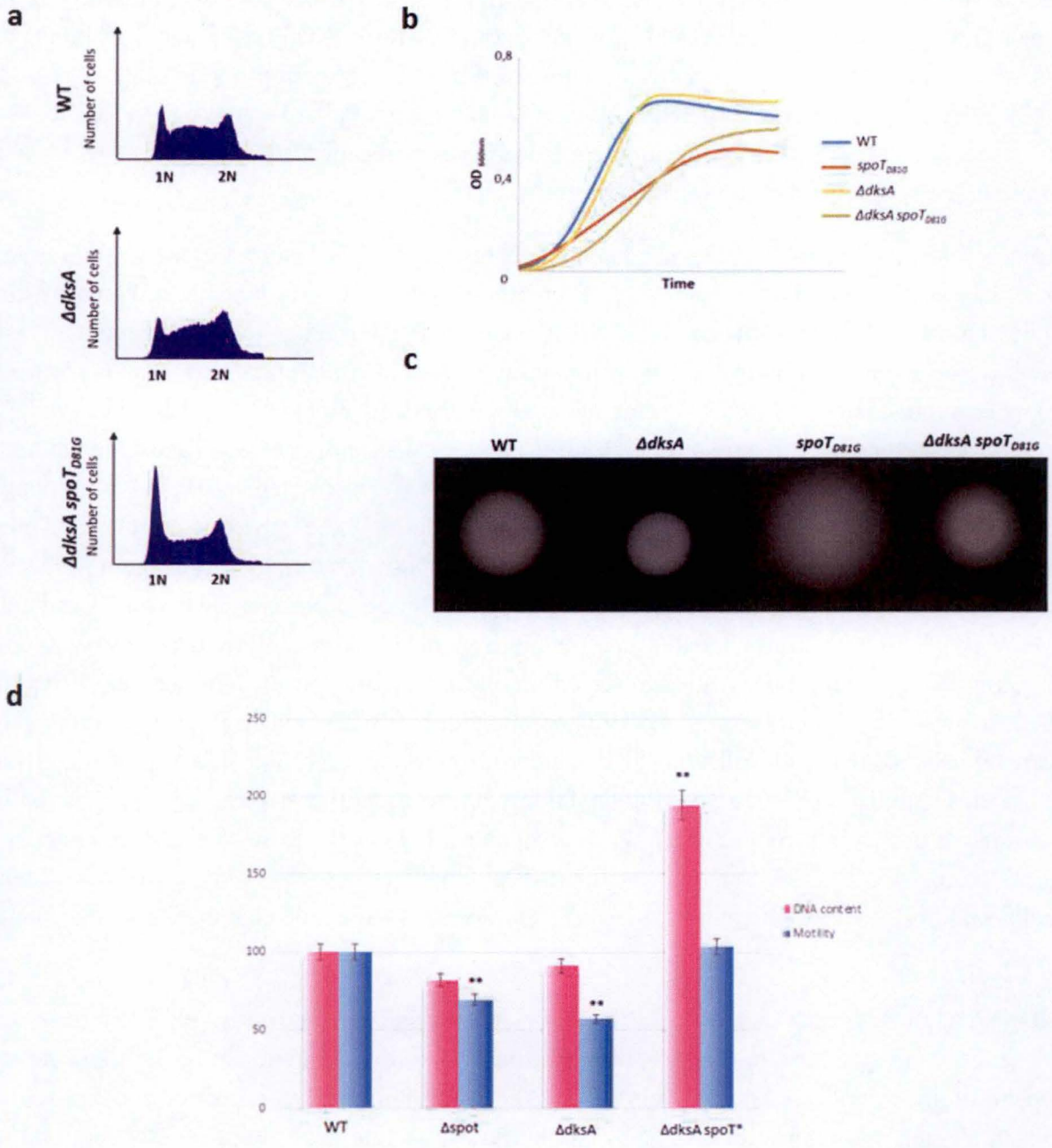

Figure 4 | Phenotypic characterization of *dksA* loss-of-function mutant . DNA content (**a**), growth (**b**), and motility (**c**) of wild-type (WT), *ΔdksA*, *spoT*$_{D81G}$ and *ΔdksA spoT*$_{D81G}$. (**d**) Motility and G1 proportion were measured and normalized to the WT (100 %). Error bars = s.d.; n=3. Statistically significant differences by Anova-test in comparison with the WT are indicated as *P<0.05% and **P<0.01% (n=3).

III. Δ*dksA* does not suppress all the phenotypes displayed by (p)ppGpp$^+$ strain

As DksA is conserved in *C. crescentus* and has been shown to stabilize binding of (p)ppGpp to the site 2 of RNAP, we checked whether DksA participates to the (p)ppGpp-dependent cell cycle control. To this end, we first created and characterized a loss-of-function mutant of *dksA* (Δ*dksA*). We found that the deletion of *dksA* did not change the G1 proportion or the growth neither in an otherwise wild-type nor in a $spoT_{D81G}$ background (**Figure 4a-b**). However, the motility was reduced in the absence of *dksA* in both backgrounds (**Figure 4c**). These results suggest that DksA is not required for the (p)ppGpp-dependent control of cell cycle, at least in the conditions used here. It is noteworthy that without *dksA*, (p)ppGpp could still efficiently bind to RNAP, should it be only on the site 1. If it is the case, the reduction of motility observed when DksA is absent might come from a down-regulation of transcription of motility genes under (p)ppGpp control. Indeed, it has been proposed that the presence of two (p)ppGpp-binding sites on RNAP would allow promoters to be sensitive different levels of (p)ppGpp, suggesting that motility genes would be more sensitive to (p)ppGpp than cell cycle genes.

IV. *rpoC* mutants that cannot bind (p)ppGpp

As stated above, both (p)ppGpp-binding sites are well conserved in *C. crescentus*. In order to evaluate the importance of transcriptional control in the phenotypes displayed by (p)ppGpp$^+$cells, we sought to construct RNAP mutants unable to bind (p)ppGpp. (p)ppGpp interacts with the β' subunit of RNAP (encoded by *rpoC*) and mutations inactivating each of these sites have been described in the literature (Ross et al, 2016). Thus we constructed molecular tools allowing replacement of the wt allele of *rpoC* by mutants unable to bind (p)ppGpp on site 1,site 2 or both sites.

Based on sequence alignments (**Figure 5a**), we decided to use mutations K615A/D622A to inactivate site 1 and N680A/K681A to inactivate site 2, all of them already shown to efficiently suppress (p)ppGpp binding on *E. coli* RNAP. As a first step to construct these molecular tools, the wt *rpoC* allele was amplified by PCR using oligonucleotides 1551/1552 (4.2 kb) and this PCR fragment was cloned into the pNPTS138 vector. Then three DNA fragments (750 bp) encompassing each or both of the mutations sets[3] were synthesized. The corresponding fragment in the pNPTS138-$rpoC^{WT}$ was replaced by each of these three mutated fragments by digestion with *Bgl* II and *Sca* I (both sites surrounding mutations, **figure 5b**).

[3] Site 1*: $rpoC_{K615A/D622A}$, Site 2*: $rpoC_{N680A/K681A}$, Sites 1-2*: $rpoC_{K615A/D622A/N680A/K681A}$

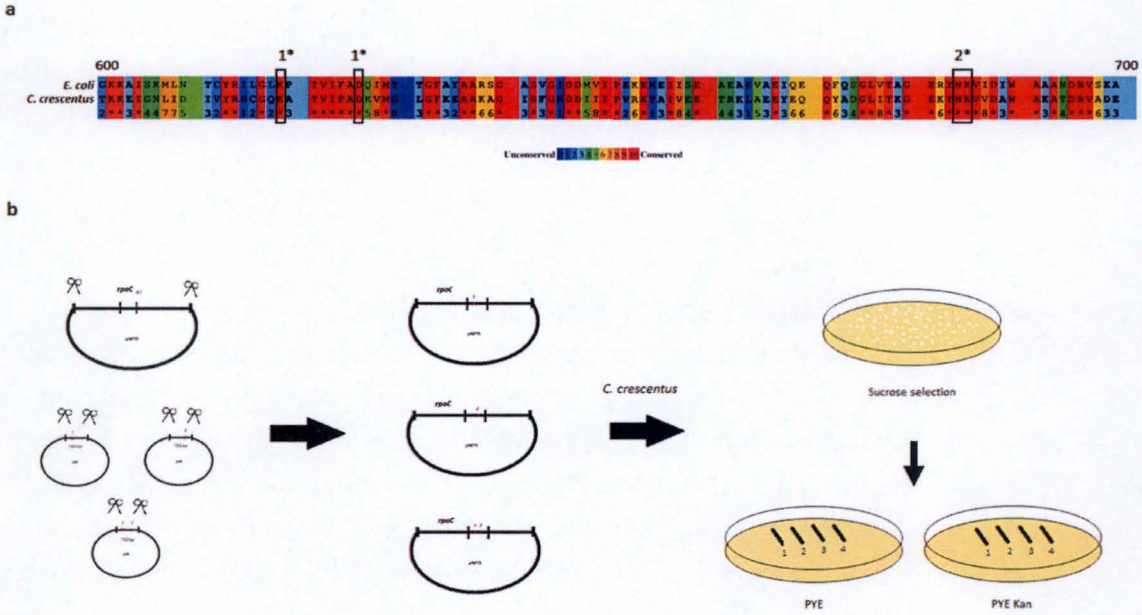

Figure 5| Conservation of two (p)ppGpp-binding sites on RNAP. (**a**) Alignment of the *rpoC* region (aa from 600 to 700 containing both (p)ppGpp-binding sites) from *E. coli* and *C. crescentus* (performed by PRALINE). (**b**) Molecular procedure followed to get (p)ppGpp-binding mutants of RNAP in *C. crescentus*.

Next we inserted mutations into the chromosome of *C. crescentus* by using a 2-step recombination procedure. The first step consists to insert each of the 3 recombinant plasmids at the *rpoBC* locus of different *C. crescentus* (WT and $spoT_{D81G}$) by selecting integration events on PYE + Kan[4] plates. Then the different strains has be cultivated overnight in PYE medium without any selection to allow the excision of the plasmids. The pNPTS138 vector harbors also a gene coding for levansucrase (*sacB*). In the presence of sucrose, SacB accumulates levan into the periplasm, which is lethal in *C. crescentus*. This plasmidic tool allows thus to counterselect cells that still have the plasmid, to keep only those which have excised the plasmid. Therefore, the overnight cultures have been plated on PYE plates supplemented with 3% of sucrose. The final steps consisted to check that clones resistant to sucrose (Suc^R) have well lost the pNPTS138 plasmid by streaking them on PYE + Kan plates and looking for kanamycin-sensitive clones (Kan^S) (**Figure 5b**), and then to test if the mutations remained into the genome after excision of the plasmid by performing discriminative PCR on Kan^S clones. Unfortunaltely, no Kan^S clones, neither in a WT nor in a $spoT_{D81G}$ background, were obtained after sucrose selection despite several attempts. In fact the sucrose selection was very inefficient in comparison to what is regularly done in the lab, suggesting that a mutation into *sacB* has been selected in the three recombinant pNPTS138, very likely during the first steps of the cloning procedure (in the pNPTS138-$rpoC^{WT}$, the ancestor of the three recombinant plasmid). We are currently redoing the cloning procedure to have fully functional recombinant pNPTS138 plasmids.

[4] The pNPTS138 vector harbors a gene conferring resistance to kanamycin

Conclusion & perspectives

Conclusion & perspectives

Here we found that (p)ppGpp showed to modulate the cell cycle through transcription regulation and proteolysis, at least for CtrA. It is likely that other post-transcriptional or translational mechanisms are also involved in this complex control since many effects of (p)ppGpp remain to be explained.

Concerning the link between (p)ppGpp and transcription, we were working on two complementary mutants. From our spontaneous mutation selected in *rpoB*, we had concluded that, even if RNAP was targeted, effects observed in strains overproducing (p)ppGpp do not all rely on transcription. For instance, CtrA and DnaA abundance in $rpoB_{T561A}$ and $spoT_{D81G}$ are different. This could indicate that (p)ppGpp-dependent regulations of CtrA and DnaA abundance do not use transcription and, at least for CtrA, we found that (p)ppGpp slows down its ClpXP-dependent proteolysis. Given the similarity between phenotypes (increase of G1 proportion, motility and doubling time), it is likely that mutation T561A in *rpoB* induces a specific conformation of the RNAP very close to the one observed upon (p)ppGpp binding. In a near future, transcripts whose levels are known to be regulated by (p)ppGpp should be measured in $rpoB_{T561A}$, for example by RT-qPCR. However further characterization of this mutant is not easy essentially because suppressive mutations appear frequently. These mutations can be reversion of T561A or other intragenic (in *rpoB*), or even extragenic (in *rpoA* and *rpoC*) (Brandis et al, 2012). That is why we decided to create a RNAP mutant having the opposite effect, that is unable to bind (p)ppGpp. Thanks to data freshly published (Ross et al., 2016) describing mutations in *rpoC* that prevent (p)ppGpp binding to RNAP in *E. coli*, we constructed tools to inactivate either each or both (p)ppGpp binding sites. Although mutated residues were highly conserved between *E. coli* and *C. crescentus*, molecular tools to incorporate them into the genome of *C. crescentus* was not easy. Indeed, the system we choose based on sucrose selection has the inconvenience to accumulate mutation in the *sacB* gene indispensable for the final selection step. As a consequence, we got no Kan^S candidates, that are clones which have excised the plasmid from the chromosome by letting (or not) the mutations. To solve this problem, we are currently redoing plasmidic constructs by checking the functionality of *sacB* at each step. Since *Ross et al.* showed that the major part of (p)ppGpp's effects on transcription is attributed to site 2 (DksA-dependent), at least in *E. coli*, it is possible that it is also the case in *C. crescentus*. However, the fact that the deletion of *dksA* does not mitigate $(p)ppGpp^+$ phenotype indicates that site 2 would not be the most important one. Indeed, Δ*dksA* in E. coli phenocopies mutated site 2.

These (p)ppGpp-binding site mutants will be a fantastic tool to study the real and direct impact of (p)ppGpp in controlling transcription. Indeed, we could first check whether direct (p)ppGpp-binding to RNAP is involved in phenotypes observed upon accumulation of the alarmone. In addition, we could compare the specific binding sites of RNAP (wt and mutants) onto the chromosome of *C. crescentus*, idendified by ChIP-SEQ, in background producing or not (p)ppGpp (Δ*spoT* and $spoT_{D81G}$).

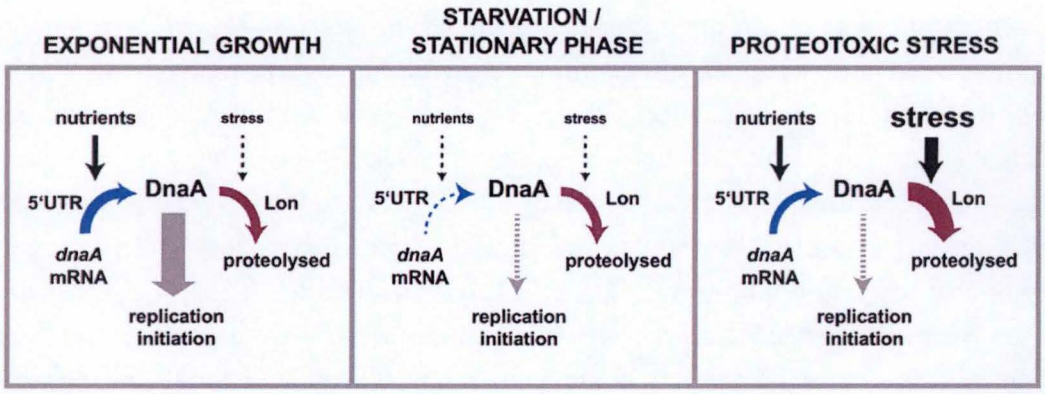

Figure 1 | Dynamic control of DnaA abundance and DNA replication in response to environmental inputs. The synthesis and the degradation of DnaA are both subject to control mechanisms that respond to environmental changes. Changes in nutrient availability modulate the rate of DnaA synthesis by a mechanism involving the 5'UTR. Changes in the global protein folding state impact the rate of DnaA degradation by the protease Lon. During exponential growth high levels of nutrients promote translation of DnaA. Although DnaA is constantly degraded, the rate of synthesis is high enough to allow for the accumulation of DnaA and DNA replication initiation. In starvation and stationary phase conditions lower amounts of nutrients cause the translation rate of DnaA to decrease. Because DnaA degradation continues at the same rate as in exponential phase, DnaA is rapidly cleared leading to a cessation of DNA replication. In proteotoxic stress conditions, for example chaperone depletion or thermal stress, nutrients are still available and drive DnaA synthesis. However, Lon-mediated DnaA degradation is stimulated in these conditions leading to the clearance of DnaA and a G1-arrest. From *Leslie et al.*; 2015.

Indeed, this experiment should help to differentiate among the promoters bound by RNAP upon (p)ppGpp accumulation, those whose regulation relies on direct (p)ppGpp-binding to RNAP. But why RNAP has two (p)ppGpp-binding sites? The best explanation would be that one site could be bound first at low (p)ppGpp concentration whereas the other site could be less sensitive and would required higher concentration of (p)ppGpp. It has been shown (p)ppGpp intracellular concentration vary widely from 10-100µM (steady-state growth) to 1mM (after starvation) and has to be distributed – through unknown mechanism. In support of this hypothesis, a mutant lacking site 2 was more defective in recovering from severe nutritional shifts. Nevertheless, deletion of *C. crescentus* DksA did not showed severe defects as seen in *E. coli* and our data do not suggest DksA requirement for (p)ppGpp-dependent control of cell cycle. Our results suggest either that site 1 is the most important (p)ppGpp-binding site or that (p)ppGpp can still bind RNAP on site 2 despite the absence of DksA. However this latter hypothesis is unlikely since site 2 is at the interface between the ß' subunit of RNAP and DksA, meaning that the pocket is formed by the interaction between both proteins.

Even if (p)ppGpp-binding sites are crucial, other aspects have to be taken into account. Sites 1 and 2 are strongly different since signaling through site 2 should integrate both (p)ppGpp concentration and occupancy of RNAP with DksA. In addition, these two sites may respond at different (p)ppGpp concentrations. Likewise, even DksA concentration is quite stable, it can encounter competition from other transcription factors. For example, changes in GreA and GreB (transcription elongation factors interacting with the ß' subunit of RNAP) levels, could potentially affect DksA-binding to RNAP. Still, these factors do not act synergistically with (p)ppGpp and are too weakly expressed to induce direct rRNA transcription regulation but can prevent DksA binding. Finally, (p)ppGpp binding to one site could change the sensitivity of the second site for (p)ppGpp and/or associated factors. For example, rifampicin-binding site is located on β' subunit of RNAP but several mutations in the ß subunit (far from the rifampicin-binding site) have been described, such as the one shown here. Likewise, RNAP conformation induced by *rif* mutations is able to suppress phenotypes of a strain (p)ppGpp^0 (Xu et al.; 2002).

Beside transcription control, our results suggest that (p)ppGpp plays also a role in controlling abundance of DnaA and CtrA cell cycle regulators. Indeed, (p)ppGpp did affect DnaA abundance by decreasing protein levels. Deletion of proteases responsible for DnaA removal did not restore DnaA levels indicating that DnaA proteolysis is not regulated by (p)ppgpp. The slower DnaA turnover in a (p)ppgpp^0 can indeed not explain the clearance of DnaA in a (p)ppgpp$^+$. On the contrary, (p)ppGpp has showed to stabilize CtrA protein at least by inhibiting its ClpXP-dependent proteolysis. We should therefore check whether (p)ppGpp could directly bind ClpXP or one of the adaptors involved in CtrA degradation, CpdR, PopA or RcdA. Interestingly, it has been shown that another hyperphosphorylated guanosine, the cyclic-di-GMP, binds to PopA and that this binding is required to properly degrade CtrA *in vivo*. Indeed, without cyclic-di-GMP PopA cannot deliver CtrA to ClpXP and CtrA is no longer degraded.

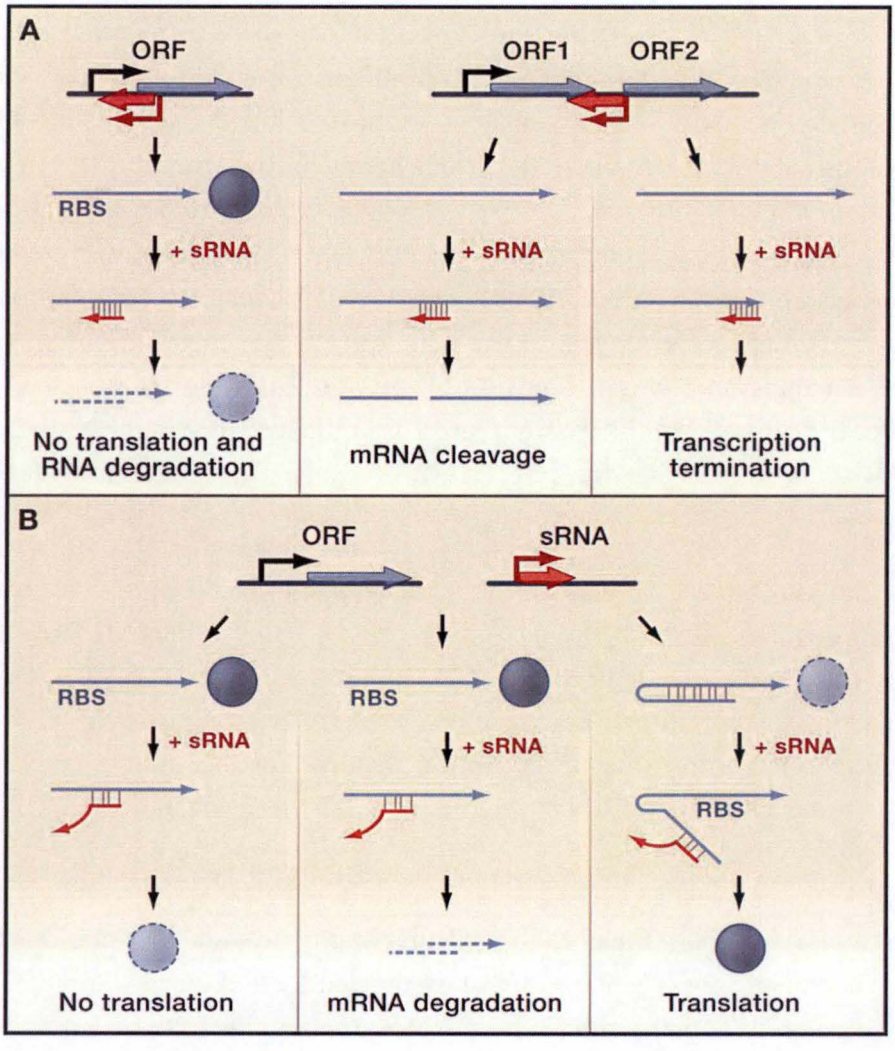

Figure 2 | Gene Arrangement and Regulatory Functions of Base Pairing Regulatory RNAs. (a) Two possible configurations of *cis*-encoded antisense sRNAs (red) and their target RNAs (blue) which share extensive complementarity. (Left panel) An sRNA encoded opposite to the 5' UTR of its target mRNA. Base pairing inhibits ribosome binding and often leads to target mRNA degradation. (Right panels) An sRNA encoded opposite to the sequence separating two genes in an operon. Base pairing of the sRNA can target RNases to the region and cause mRNA cleavage, with various regulatory effects, or the sRNA can cause transcriptional termination, leading to reduced levels of downstream genes **(b)** Genes encoding *trans*-encoded antisense sRNAs (red) are located separate from the genes encoding their target RNAs (blue) and only have limited complementarity. *Trans*-encoded sRNA can act negatively by base pairing with the 5' UTR and blocking ribosome binding (left panel) and/or targeting the sRNA-mRNA duplex for degradation by RNases (middle panel). *Trans*-encoded sRNA can act positively by preventing the formation of an inhibitory structure, which sequesters the ribosome binding site (RBS) (right panel). From *Waters & Storz* (2009).

Thus (p)ppGpp could compete with cyclic-di-GMP to bind PopA or binds to it at another site. Alternatively, (p)ppGpp might interfere with cyclic-di-GMP levels by regulating the activity of the enzymes synthesizing it or GTP levels.

As the *rpoB*$_{T561A}$ mutant mimicking (p)ppGpp-binding does not modulate CtrA and DnaA levels, DnaA and perhaps CtrA also should be regulated at the post-transcriptional level. Interestingly, DnaA abundance is regulated by post-transcriptional mechanisms involving translation regulation. Indeed, *dnaA* contains a long 5' untranslated leader region, shown to alter *dnaA* expression during exponential phase (Leslie et al. 2015). Moreover this 5'-UTR does affect *dnaA* expression in stationary phase. Translation of dnaA mRNA significantly diminishes at the transition from exponential to stationary phase. By decreasing translation of DnaA without interfering with its degradation by Lon, DnaA levels rapidly decrease leading to a G1 arrest (**Figure 1**). However, this regulation still works in Δ*spoT* cells, suggesting that (p)ppGpp is not strictly required for mediating this translational control. Nevertheless, the alarmone could stimulate and it would be interesting to test if the 5'-UTR-mediated control of DnaA is enhanced in (p)ppGpp$^+$ cells.

In addition to transcriptional control, prokaryotes have also evolved another mechanism to adjust cell cycle progression in response to environmental cues – *trans*-encoded small non-coding RNAs (*trans*-sRNAs). A *trans*-sRNA might also be involved in the *dnaA* regulation and (p)ppGpp could interfere with this sRNA Prokaryotic trans-sRNAs are functional analogs of eukaryotic siRNAs and miRNAS in their ability to post-transcriptionally control gene expression by modulating mRNA translation and stability. This post-transcriptional mechanism relies on short and often imperfect base-pair interactions within the 5'-UTR of the target mRNA which leads to the formation of a sRNA-mRNA duplex (**Figure 2**). sRNA range in size between 50 and 250 nucleotides and have target sequences ~ 6–7 nucleotides that are usually located in 5′ untranslated regions (UTRs). The inhibition of translation initiation mediated by sRNA blocking the ribosome binding site also leads to mRNA cleavage by RNases and promotes Rho[5]-dependent premature transcription termination. Regulatory RNAs can directly sense small metabolites, ions, temperature of even pH and respond to it by altering transcription, translation or RNA stability. Indeed, bacteria express commonly sRNAs under stress conditions which contribute to the rapid cellular response and adaptation. sRNAs actively participate to control physiological processes such as metabolism, chemotaxis, biofilm formation, virulence and pathogenesis as well.

In Gram-negative bacteria, activity of most sRNAs is dependent on their binding to Hfq, which favors sRNA–mRNA annealing and protects unpaired sRNA from degradation by RNases. Hfq is an important cofactor for the post-transcriptional regulatory of many sRNAs that mediate adaptation to environmental conditions. Occasionally, Hfq can also directly inhibit translation initiation, guided by sRNAs and occluding the ribosome binding.

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[5] Rho is a multi-functional termination factor involved in transcription regulation. Its ATPase activity is stimulated by RNA binding and leading to dissociation of the transcription elongation complex.

However, post-transcriptional regulation by sRNAs do not only result in inhibition. For instance, some sRNA are known to prevent premature transcription termination. In some cases, sRNA stimulate mRNA translation through conformational rearrangements (Sedlyarova et al, 2016).

Moreover, a same sRNA can be degraded or reused and its fate will depend on the mRNA target(s). Its abundance has also its importance since a "threshold linear response" requires that the rate of sRNA synthesis exceeds the rate of mRNA synthesis. In this way, accumulation of mRNA leads to sequestration of inactivation of the sRNA.

As a same sRNA has several mRNA targets, if one of these mRNA targets shows higher production, that will lead to deregulation of the other targets since sRNA will not be enough available (Sedlyarova et al.; 2016). Such an interplay of transcription regulation and crosstalk between RNAs provides a mechanism allowing communication among different regulatory pathways in charge of many processes control – as observed in bacteria. Through competition, RNAs can cross-regulate translation, transcription and/or stability of other RNAs.

Robledo et al. have revealed a role for trans-sRNA EcpR1 in the *S. meliloti* cell cycle regulation. *Trans*-sRNAs are known to contribute to a rapid cellular response to various changes but have not directly related to the cell cycle regulation, until now. Interestingly, it is confirmed that this *trans*-sRNA cell cycle targets are *dnaA* and *gcrA* mRNAs, this latter being a positive regulator of *ctrA* transcription. Also, 27 sRNAs were identified in *C. crescentus* to exhibit a differential response to nutritional or stationary phase challenge. Additionally, *C. crescentus* possesses an ortholog of the Hfq RNA-binding protein found in many bacterial species. Several of these sRNAs have shown to bind Hfq. Since a *hfq* mutant shows impaired survival under several different stress conditions, sRNAs seem to be involved in environmental adaptation. Furthermore, expression of four sRNA was cell cycle regulated. Regulatory motifs were also identified at some promoter regions of many genes implicated in both cell cycle control and swarmer-to-stalked cell transition. Evidence supports a role for CtrA in control of a precise sRNA with (i) presence of a consensus CtrA biding motifs upstream of the sRNA site, (ii) accumulation of this sRNA only in presence of CtrA and (iii) binding of CtrA in the promoter region. sRNAs may play a significant role in cell cycle regulatory pathways of *C. crescentus* (Landt et al. 2008). Testing the involvement of sRNA(s) in the (p)ppGpp-dependent regulation if CtrA and/or DnaA would thereby be interesting.

Materials & methods

Material & methods

I. Strains & plasmids

All strains and plasmids used in this thesis are described in Annexe 1.

II. Growth conditions.

a. Escherichia coli

Escherichia coli were grown in LB broth medium at 37°C with shaking (200-250RPM). MT607-pRK600 (RH 319) was used for triparental matings.

E. coli strains are stored at -80°C :
- Grow cells in 10ml LB broth (supplemented with appropriate antibiotics if required) overnight at 37°C with shaking (200-250RPM)
- Mix 900µl of saturated culture and 100µl of DMSO, vortex and freeze.

b. Caulobacter crescentus

Caulobacter crescentus were grown in PYE (complex) or M2G (minimal) media at 30°C with shaking (200-250RPM). NA1000 (RH50) was used as wild-type reference strain depending on the experiments. NA1000 has doubling times of about 90 min in PYE and 130 min in M2G.

C. crescentus strains are stored at -80°C:
- Grow cells in 5ml PYE (supplemented with appropriate antibiotics if required) overnight at 30°C with shaking (200-250RPM).
- Mix 900 µl of saturated culture and 100µl of DMSO, vortex and freeze.

Mediums, buffers and solutions are described in Annexe 2.

III. Bacterial genetics

a. Escherichia coli

Transformation

- **Electroporation**

Preparation of electrocompetent cells

- Inoculate a fresh colony of RH783, RH93 into SOB medium and grow overnight at 37°C.
- Transfer 1ml of the o/n culture into 1L SOB medium
- Grow at 37°C until OD550 is 0,8 (about 2 to 3 hr).
- Harvest cells by centrifugation at 5000rpm (2600 x g) at 4°C for 10'.
- Resuspend cells in 500ml ice-cold WB [or sterile distilled water].
- Repeat 5000 rpm/ 10' spin and wash cells by resuspending it in 500ml ice-cold WB.
- Repeat 5000 rpm/ 10' spin and resuspend cells in ice-cold WB so that the final volume is about 4ml.
- Freeze cell in 55µl aliquots in 1.5ml tubes using a dry ice-ethanol bath and store at -80°C.

Electroporation

- Thaw frozen cells on ice.
- Chill 0.2 cm electroporation cuvette on ice.
- Add 2-10µl plasmid DNA (dialyzed 20' on 0,022µm filter) to 50µl of cells.
- Set up the electroporator at 200 ohms, 25 µFD, 2.5 kV.
- Place the cuvette in electroporation chamber and hold on both buttons. The pulse length should be ~4-5msec.
- Immediately add 1ml superbroth for *E. coli* and mix.
- Transfer cells to sterile tube and incubate 1 hr at 37°C.
- Plate dilutions on selective media (LA plates supplemented with appropriate antibiotics) and incubate o/n at 37°C.

Cleaning electroporation cuvettes

- Rinse cuvettes several times in deionized water.
- Rinse with EtOH to kill bacteria. Discard EtOH.
- Irradiate with UV light 15 min (destroys DNA).
- Rinse with EtOH and dry at room temp or in 55°C oven. Cuvettes canbe used 8-10 times.

- **Heat-shock transformation**

Preparation of competent cells

- Inoculate a fresh colony of RH93, RH783 into LB medium and grow overnight at 37°C or at permissive temperature.
- Transfer 1ml of the o/n culture into 1L LB medium.
- Grow at 37°C until OD $_{550}$ is 0.4-0.5 (about 2 to 3 hr); then place it on ice for 20'.
- Harvest cells by centrifugation at 5000 rpm (2600 x g) at 4°C for 10'.
- Resuspend cells in 200ml pre-chilled 50mM $CaCl_2$ and keep it on ice for 30-45'.
- Repeat 5000 rpm/ 10' spin and resuspend cells in 7.0 ml 50mM $CaCl_2$/15% Glycerol.
- Freeze cells in 100µl aliquots in 1.5 ml tubes using a dry ice-ethanol bath and store at -80°C.

Transformation

- Thaw frozen cells on ice.
- Mix 2-10µl plasmid DNA to 100µl cells.
- Place the tube on ice for 30-45'.
- Place the tube at 42°C for 2' (Heat shock).
- Immediately add 1ml LB or superbroth for *E. coli*, mix and incubate 1 hr at 37°C.
- Plate dilutions on selective media (LA plates supplemented with appropriate antibiotics) and incubate o/n at 37°C.

b. Caulobacter crescentus

Transduction with CR30

CR30 phages require an intact S-layer to infect Caulobacter, meaning that all rsaA- strains cannot be infected (and transduced) by CR30.

Phage lysate preparation

- Inoculate a fresh colony of the donor strain into PYE medium and grown overnight at 30°C.
- Transfer 150µl of the o/n culture into 5ml PYE medium and grow at 30°C until OD_{660} is 0.4-0.7 (about 5 to 6 hr).
- Dilute CR30 stock lysate prepared on NA1000 (LHR2) from 10-2 to 10-5 in *Caulobacter* Phage Buffer (CPB).
- Mix 200µl of each phage dilution and 200µl of the donor strain and incubate 10' at RT.
- Transfer the mix to 4ml of prewarmed (~50°C) PYE Top Agar, vortex briefly and pour out onto a prewarmed (~30°C) PYEA plate.
- Incubate o/n at 30°C without inverting the plates.
- After o/n incubation at 30°C or at permissive temperature, choose plate(s) dilutions on which phages got confluent lysis.
- Add ~2ml of chloroform on the top of the topagar and let it evaporate under the chemical hood.

- When the chloroform is completely evaporated, add 5ml of CPB onto plates and gently shake for >2 h at RT.
- Transfer the liquid into a glass Corex tube and centrifuge at 6500 rpm (5000 x g) at RT for 5'.
- Transfer the supernatant into a glass flask with a screw cap dedicated for phage lysates.
- Add 150µl of chloroform, vortex and let si o/n at 4°C. If you need to use the CR30 lysate immediately, centrifuge at 6500 rpm (5000 x g) at RT for 5' and use supernatant for transduction.
- If required, determine titer of lysate by infecting NA1000. Alternatively, assume that the titer is about 10^8-10^9 plaque-forming –units (pfu)/ml.

Transduction

- Inoculate a fresh colony of the recipient strain into PYE medium and grow overnight at 30°C or at permissive temperature.
- Transfer 150µl of the o/n culture into 5ml PYE medium and grow at 30°C or at permissive temperature until OD_{660} is 0.4-0.7 (about 5 to 6hr).
- Inactivate CR30 phages (50µl/transduction in small Petri dish) by exposing it to UV (Stratalinker for 2').
- Mix 25µl of the CR30 lyste with 475µl of PYE and 500µl of the recipient strain.
- Incubate 2h at 30°C or at permissive temperature w/o shaking.
- Harvest cells by centrifugation at 9000 rpm for 1' and resuspend the pellet in 100µl PYE.
- Plate 100µl on selective media (PYEA plates supplemented with appropriate antibiotics) and incubate 2-3 days at 30°C or at permissive temperature.
- Streak transductants at least once on PYEA plate supplemented with appropriate antibiotics to get rid of residual CR30 phages from plates.

Mating

Triparental mating

- Inoculate a fresh colony of the donor strain (E. coli strain deficient for conjugation, harboring the plasmid to transfer) into LB medium supplemented with appropriate antibiotics, and the Helper strain (RH 319) in LB Cam; and grow overnight at 37°C or at the permissive temperature.
- Inoculate a fresh colony of the recipient strain into PYE medium and grow overnight at 30°C or at permissive temperature.
- Mix 50µl of the donor strain, 50µl of the Helper strain and 900µl of the recipient strain.
- Harvest cells by centrifugation at 9000 rpm (7600 x g) at RT for 2'.
- Remove supernatant, add 1ml of fresh PYE medium and wash the pellet by centrifugation at 9000 rpm (7600 x g) at RT for 2'.
- Remove most of the supernatant and resuspend the pellet with about 50µl.
- Spot the drop on a PYEA plate and incubate at 30°C or at permissive temperature for 4h (replicative plasmid) or o/n (non replicative plasmid).

- Scrap the growing drop, streak it on PYEA plate supplemented with Nalidixic acid and appropriate antibiotics and incubate at 30°C or at permissive temperature for 2-3 days.
- Streak 2-3 colonies on fresh PYEA plates supplemented with appropriate antibiotcs (w/ Nal) and incubate overnight at 30°C or at permissive temperature for 1-2 days.

IV. Bacterial physiology

a. C. crescentus

DNA content with FACS

Fixation of cells

- Inoculate a fresh colony of your strain into 5ml complex or synthetic medium and grow overnight at 30°C or at permissive temperature.
- Transfer 250µl of the o/n culture into 5ml fresh medium and grow at 30°C at permissive/restrictive temperature until OD_{660} is 0.4-0.6.
- If you want to determine the proportion of heterogenous population in G1 phase, add Rif (20µg/ml) and incubate for 3h at 30°C. If you want to determine the proportion of hetegenous population in the 3 different phases (G1, S & G2), skip this step.
- Transfer 1ml of cells into 9ml of ice cold 77% Ethanol and mix well.
- Store at -20°C for up to 2 months.

Staining of cells

- Wash 2ml of the fixed cells in 1 ml FACS Staining Buffer.
- Harvest cells by centrifugation at 8000 rpm for 2' and resuspend the pellet in 1ml FACS Staining Buffer containing 0.1mg/ml RNaseA and incubate at RT for 30'.
- Harvest cells by centrifugation at 8000 rpm for 2' and remove supernantant.
- Resuspend the pellet in 1ml FACS Staining Buffer containing 0.5 µM Sytox Greeen and incubate at RT in the dark for 5'.
- Analyze samples in flow cytometer (FACS SCAN, Argon Laser excitation at 488nm).
- The optimal cell number for flow cytometer: ~10^7/ml. if necessary dilute the cells 1:10 in staining buffer containing 0.5 µM Sytox Green. Always analyze freshly stained cells.

FACS Procedure

- Turn on the FACS by following the instructions indicated on the cytometer.
- Turn on CellQuest Pro software.
- Choose Connect to Cytometer from the Acquire menu. The Acquisition view of the Browser appears.
- Change the directory to store your data.
- Set instrument settings by opening the instrument settings file. Once loaded, click first "Set" button then on "Done" button.

- Check the settings in Detector/Amp:

Detector	Voltage	AmpGain	MODE	Gate
FSC	E01	1.00	LOG	No
SSC	631	1.00	LOG	No
FL1 (Alexa 488)	613	1.00	Ln	200-600

- Draw an Histogram Plot and a Dot Plot in the document.
- Open the Inspector Histogram/Dot Plot (Plots/Format Histogram) and set the following parameters :

Plot	Plot Type	X Parameter	Y Parameter
Histogram	Acquisition	FL1-H 1024	Counts
Dot 1	Acquisition	FSC-H 1024	FL1-H 1024
Dot 2	Acquisition	FSC-H 1024	SSC-H 1024

- Load a reference sample (e.g wt +/- Rif) and place the flow cytometer in RUN (low) mode.
- Click Acquire in the upper panel of the Browser. Note that the Setup check box in the Acquisition view of the Browser is checked. This allows you to click Acquire and view real-time acquisition display without saving a data file.
- Draw a region of interest in the Dot Plot 1 (to gate on the population of interest) by clicking the Square-region tool in the tool palet. Usually gate from 200 to 600 for the FL1-H 1024 signal.
- Go back to the Inspector Histogram/Dot Plot (Plots/Format Histogram) and choose Gate G1=R1 from the gate pop-up menu.
- Set up the Acquisition & Storage settings (Acquire/ Acquisition é Storage settings) :
    - Acquire and store G1=R1.
    - $5 \times 10^4 - 1 \times 10^5$ events for asynchronous populations and $1 \times 10^4 - 2.5 \times 10^4$ events for synchronous populations.
- Collect the data by using the Acquire button. Don't forget to uncheck the Setup check box in the Acquisition view of the Browser to store the data.
- Wash and turn off the FAS by following the instructions indicated on the cytometer.

Motility assay

Swarming motility test

- Inoculate a fresh colony of your strain into 5ml PYE or M2G medium and grow overnight at 30°C or at permissive temperature.
- Pick up a toothpick and dip it into the o/n culture.

- Stab toothpick into an appropriate spot on PYE Swarmer Agar plate (0.3% agar). Do not touch the bottom of the plate with your toothepick because cells may diffuse between agar and plastic.
- Do not forget to include positive (RH50) and negative (RH217) controls. It is best to do also all strains on the same plate as well as in replicate twice more to ensure reliable results.
- Store plates at 30°C or at permissive temperature, with the lids up, for 2-3 days.
- Measure the diameter of bacterial growth surrounding each stab- using a software (ref 16).

Growth & viability

Bioscreen

- Inoculate a fresh colony of your strain into 5ml PYE and grow overnight at 30°C or at permissive temperature.
- If the medium tested is not PYE, then dilute the o/n culture into M2G or whatever you would like to test for growth and incubate overnight at 30°C or at permissive temperature.
- Dilute the o/n culture into 2ml of medium so that final OD_{660} is ~0.03 by using the following formula: **x (µl) = 0.03/OD_{660}** (o/n culture).
- Transfer 200µl of bacterial suspension per well into 4-8 wells of a 100-wells plate Bioscreen.
- Use the first and last lines and rows as blank (medium).

VI. Molecular Biology

a. DNA

Amplification of DNA by PCR: PCR for screening

Mix|	Program	
21,2 µL H2O	98°C – 5'	
6 µL Green Taq polymerase Buffer (5x)	98°C – 30''	|
1,5 µL DNTPs (5mM)	52-66°C* – 30''	| 30X
0,6 µL primer 1 (20 µM)	72°C – a x 1'	|
0,6 µL primer 2 (20 µM)	72°C – 10'	
0,2 µL Taq polymerase (1U)		

*depends on the melting temperature of your primers. Classically use 54°C for PCR done on *E. coli* and 58°C when DNA is amplified from *C. crescentus*.

a: # of kb (1' for a 1000 bp amplicon for example).

Amplification of DNA by PCR: PCR for cloning

Mix	Program	
20 µL H2O	98°C – 10''	|
25 µL PrimeStar GC Buffer (2x)	52-68°C – 5''	| 30X
2 µL DNTPs (200µM)	72°C – a x 1'	|
1µL DNA*	or	
0,75 µL primer 1 (20µM)	98°C – 10''	|
0,75 µL primer 2 (20µM)	68°C – a x 1'	| 30X
0,5 µL PrimeStar DNA Polymerase (1,25U)		

*Can be replaced by 1µl of colony heat-inactivated or 1µl of H2O and 1 colony of the strain freshly streaked on plate. For the last, a step of lysis (98°C during 2') may be required.

a: # of kb (1' for a 1000 bp amplicon for example).

Rules for primers construction

- Primers should have a similar theoretical TM – around 58°C for E. coli and 62°C for C. crescentus
- The GC content or each primer should be around 60%

- The 3'end of primers should not contain more than 3 G/C in the last 5 bases – and 1 or 2 G/C at the very 3' end will be favored.
- Restriction sites required to digest the restriction sites directly from the linear PCR product will be added at the 5' end of the primers.

Restriction of linear or circular DNA

Mix : final volume 10µl or 20µl

2-4 µl restriction enzymes

1 or 2 µl restriction buffer* (10X)

7-14 µl DNA

- Incubate at 37°C for 2 h.
- Purify restriction products from agarose gel using the Qiagen Gel extraction kit or clean up restriction products using the PCRapace kit. Quantify the digested PCR using the Nanodrop.

Ligation

Mix : final volume 10µl or 20µl

X µl plasmid vector^{+}

Y µl insert (digested PCR or recombinant plasmid)$^{+}$

1 µl T4 DNA ligase^{+}

2-4 µl Ligase Buffer (5X)

Z µl $H2O^{+}$

$^{+}$ x and y are determined by using the following rule:

$$\text{Mass insert (ng)} = \frac{[\text{Mass Vector (ng)} \times \text{Size Insert (bp)}] \times a^{*}}{\text{Size Vector (bp)}} \qquad *a = 3\text{-}10$$

- Incubate o/n at RT.
- Eventually digest the ligation product with a Restriction Enzyme that cuts the plasmid vector (between the 2 restriction sites used for the cloning) but not the insert.
- Transform 10µl or 20µl into RH783 competent cells.

b. Purification of linear DNA

PCR clean up

PCRapace, INVITEK

- Add Binding Buffer to the PCR reactions (250µl for PCR mix up to 50µl; 500µl for PCR mix > 50µl up to 100µl) and mix very well by pipetting or vortexing.
- Transfer the sample completely onto a Spin filter and centrifugate for 3' at 12000 rpm.
- Remove the filtrate and centrifuge again for 2' at 12000 rpm.
- Place the Spin Filter into a new 1.5 ml Receiver Tube.

- Add at least 10µl of Elution Buffer (or dH_2O) directly onto the center of the Spin Filter.
- Incubate for 1' at RT and centrifuge for 1' at 10000 rpm.

Extraction from gel

- Excise the DNA fragment from the agarose gel with a clean, sharp scalpel. Minimize the size of the gel slice by removing extra agarose.
- Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QG to 1 volume of gel (100mg ~100µl). For >2% agarose gels, add 6 volumes of Buffer QG. The maximum amount of gel slice per QIAquick column is 400mg; for gel slices >400 mg use more than one QIAquick column.
- Incubate at 50°C for 10'.
- Add 1 gel volume of isopropanol to the sample and mix.
- Place a QUIAquick spin column in a 2ml collection tube.
- To bind DNA, apply the sample to the QIAquick column and centrifuge for 1min.
- Discard flow-through and place QIAquick column back in the same collection tube.
- To wash, add 0.75 ml of Buffer PE to QIAquick column and centrifuge for 1min.
- Discard the flow-through and centrifuge the QIAquick column for an additional 1min at 13000rpm.
- Place QIAquick column into a clean 1.5 ml microcentrifuge tube.
- To elute DNA, add 50µl of Buffer EB (10mM Tris-Cl, pH 8.5) or water (pH 7.0-8.5) to the center of the QIAquick membrane and centrifuge the column for 1min. Alternatively, for increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1min and then centrifuge for 1min.

c. Proteins

Western blot

Crude extract preparation

- Inoculate a fresh colony of your strain into 5ml complex medium and grow overnight at 30°C.
- Dilute the o/n culture and grow at 30°C until OD660 is 0.3-0.5.
- Transfer 2 ml of the culture into a 2ml tube and harvest cells by centrifugation at 10000 RPM for 2'.
- Discard the supernatant and resuspend the pellet in a volume (µl) equivalent to OD660 x 200 of 1X SDS-loading buffer.
- Boil the sample 5-10' and store it at -20°C.
- Load 10-25µl per lane (electrophoresis in a 4–15% SDS–polyacrylamide gel).

Transfer

- Equilibrate acrylamide gel in transfer buffer for 20' using gentle shaker.
- Soak nitrocellulose membrane transfer buffer for 20' using gentle shaker.
- Ensure that there is not any bubble trapped

- Put the cathode and safety cover
- Run at constant voltage 14V; 3mA/cm²
- To block membrane, incubate the membrane in PBS with 0.05% Tween 20 and 5% Non-fat milk (PBS_Tween_Milk) in cold room overnight.

Immunoblot

- Discard blocking solution and add 5-10ml of diluted primary antibody in PBS_T_M and incubate 1-3h. In these cases, membranes were immunoblotted for 3h with primary antibodies : α-SpoT (1:5,000; ref. 47), α -MreB (1:5,000; ref. 46); α- DnaA (1: ; ref) and α-CtrA (1:5000 ; ref).
- Wash membrane 3-5X with PBS with 0.05% Tween (PBS_T) for 2-3 minutes each.
- Add 5-10ml of diluted mutatio antibody (1:7,500 anti-rabbit- linked to peroxidase (GE Healthcare)) in PBS_T_M and incubate 1h.
- Wash membrane 5X with PBS_T for 5-10 minutes each.

ECL detection

- Mix ECL solutions in a ratio of 1:1
- Remove PBS_T from blot and incubate membrane blot with detection reagent for 1-2' at room temperature.
- Remove membrane, wrap in Saran Wrap and expose to X-ray film before development and visualized thanks to Western Lightning PlusECL chemiluminescence reagent (Biorad) and ImageQuant LAS400 (GE Healthcare).

Annexes

Annexe 1

a. Strains

Name	Description and relevant genotype	Reference
RH319	MT607(*pro*-82 *thi*-I *hsdR17* (r-m+) *supE44 recA56*)	Evinger & Agabian, 1977
RH783	Top10 (*φ80lacZΔM15 araD139* Δ (*ara-leu*)7697 *galE15 galK16* Δ(*lac*)X74 *rpsL*(StrR) *nupG recA1 endA1 mcrA* Δ (*mrr-hsdRMS-mcrBC*)	Life Technologies
RH50	NA1000	Casadaban & Cohen, 1980
RH1752	NA1000 $spoT_{D81G}$	Ronneau et al., 2016
RH1755	NA1000 Δ*spoT*	Ronneau et al., 2016
RH2242	NA1000 Δ*rpoZ*	This study
RH2243	NA1000 Δ*dksA*	This study
RH2244	NA1000 Δ*dksA* $spoT_{D81G}$	This study
RH2245	NA1000 $rpoB_{T561A}$	This study
RH2246	NA1000 Δ*spoT* $rpoB_{T561A}$	This study
	NA1000 Δ*lon*	This study
	NA1000 Δ*lon*Δ*spoT*	This study
	NA1000 Δ*lon* $spoT_{D81G}$	This study
	NA1000 Δ*clpA*	This study
	NA1000 Δ*clpA*Δ*spoT*	This study
	NA1000 Δ*clpA* $spoT_{D81G}$	This study
	NA1000 Δ*cpdR*	This study
	NA1000 Δ*cpdR*Δ*spoT*	This study
	NA1000 Δ*cpdR* $spoT_{D81G}$	This study

b. Plasmids

Name	Description	Reference
	pNPTS138	M. R. Alley, Imperial College London (UK), unpublished
pHR750	pNPTS138-Δ*dksA*	This study
pHR857	pNPTS138-Δ*rpoZ*	This study
pHR369	pNPTS138-Δ*glnC*	Ronneau et al., 2016
	pNPTS138-*spoTD81G*	This study
	pNPTS138-Δ*rpoZ*Δ*spoT*	This study
	pNPTS138-rpoC-WT	This study
	pNPTS138-rpoC-site1*	This study
	pNPTS138-rpoC-site2*	This study
	pNPTS138-rpoC-site1/2*	This study

c. Oligonucleotides

Name	Sequence
1161	tcAAGCTTgtggttcgtcgggcttttcg
1162	tcGAATTCtgtggccgtctgCATaacgc
1163	tcGAATTCgactaagcgctgatctggcg
1164	tcGGATCCatagcccacgaacaccagcg
1167	cctaagtaactaaGGATCcggagcctatacgagtcgcg
1168	gtatccccgggtaGAATTCgcaatcttcgacggtgacgcg
1169	tcGAATTCctgcaaagcgaccgagacgg
1170	tcAAGCTTggagagcttggtgacgcc
1551	
1552	
1559	TACCGCCACTGCGGTCAGgct
1560	AAGGGGGAGAAGTACgcCgcG
1561	TACTCCTGAACGGTCAGGCC
1562	AAGGTGATCGACACCACGCC

Annexe 2

a. Mediums

For *E. coli*: LB (Luria Bertani) medium

	Liquid LB	LB agar	LB Top Agar	SOB Medium (low salts)
Bacto-tryptone	10g	10g	1g	20g
Yeast extract (Difco)	5g	5g	0,5g	5g
NaCl	5g	5g	0,5g	0,584g
Agar (Difco)	-	15g	0,7g	0,186g
dH_2O	1L	1L	0,1L	1L

For *C. crescentus*: PYE (Peptone Yeast extract) medium/ complex medium

	Liquid PYE	Solid PYE	PYE Top Agar (0.45%)	PYE Swarmer Agar (0.3%)
Bacto-tryptone	2g	2g	2g	2g
Yeast extract (Difco)	1g	1g	1g	1g
0.5 M $MgSO_4$	2ml	2ml	2ml	2ml
0.5 M $FeSO_4$	1ml	1ml	1ml	1ml
Agar (Difco)	-	15g	4,5g	3g
dH_2O	1L	1L	1L	1L

For *C. crescentus*: synthetic medium

	M2G
20x M2 salts	50 ml
20% Glucose	10 ml
0.5 M $MgSO_4$	1 ml
10 mM $FeSO_4$	1 ml
0.5 M $CaCl_2$	1 ml
dH_2O to	0.999L

b. Buffers & solutions

	Componants	Quantity
Running gel 12% (for 5 mL final volume)	H2O	1,6 mL
	Acrylamide 30%	2 mL
	Tris-HCl pH 8,8 1,5 M SDS 0,1%	1,35 mL
	APS 10%	0,05 mL
	TEMED	0,003 mL
Stacking gel (3 mL final volume)	H2O	1,7 mL
	Acrylamide 30%	0,5 mL
	Tris-HCl pH 6,8 1,5 M SDS 0,1%	0,8 mL
	APS 10%	0,03 mL
	TEMED	0,003 mL
Blocking solution	APS 10%	0,03 mL
	TEMED	0,003 mL
PBS 10X	PBS Tween 0,05%	1L
	Milk (powder)	50g
	K_2HPO_4 20mM	2,72g
	Na_2HPO_4 100mM	14,2g
	KCl 20mM	1,5g
	NaCl 1,4M	81,2g
SDS-Loading buffer 5X *4°C conservation*	1M Tris (pH 6.8)	12,5 mL (250mM)
	100% Glycerol	25mL (50%)
	SDS	5g (10%)
	Bromophenol blue	Few grains
	B-mercaptoethanol	Up to 50 mL

Z buffer	Na_2HPO_4	8,64g
	NaH_2PO_4	4,8g
	KCl	0,75g
	$MgSO_4.7H_2O$	0,246g
	dH_2O	To 1L
ONPG Substrate	ONPG	40mg
	Z buffer	10 ml
Na_2CO_3 1M	Na_2CO_3	21,2g
	dH_2O	0,2L
Lysis buffer	Polymyxin B	0,1g
	B-mercaptoethanol	13,5µl
	Z Buffer	5ml
1M Tris (pH 6.8, 7.5 or 8.0)	Tris	121g
	dH_2O	1L
10x PBS (pH 7.4)	NaCl	80g
	KCl	2g
	Na_2HPO_4	14,4g
	KH_2PO_4	2,4g
	dH_2O	1L
Caulobacter Phage Buffer	0.5 M Tris (pH 7.5)	2ml (10mM)
(CPB)	0.5 M $MgSO_4$	200µl (1mM)
	0.5 M $CaCl_2$	200µl (1mM)
	dH_2O	0,1L

FACS Staining Buffer (pH 7.2)	1M Tris	5ml (10mM)
Filter sterilize	0.5 M EDTA	1ml (1mM)
	1M NaCitrate	25ml (50mM)
	TritonX-100	50µl (0.01%)
	dH_2O to	0,5L
	glycine	14,4g
Transfer Buffer (TOWBIN)	Tris	3g
	Methanol	0,2L (for proteins <70kDa)
		Or 0,05L(for proteins >70kDa)
	dH_2O to	1L

c. Salts

	Componants	**Quantity**
1M NaCl	NaCl	58,4g
Filter sterilize	dH_2O	1L
1M MgCl2	$MgCl_2.6H_2O$	20,3g
Filter sterilize	dH_2O	1L
0,5M CaCl2	$CaCl_2$	5,55g
	dH_2O	0,1L
0,5M MgSO4	$MgSO_4.7H_2O$	12,3g
	dH_2O	0,1L

d. Antiobiotics & sugars

Antiobiotic/Sugar	Dissolution' solution	Stock solution (µg/µL)	Liquid LB Concentration (µg/mL)	Solid LB Concent°(µg/mL)	Liquid PYE Concent° (µg/mL)	Solid PYE Concent° (µg/mL)
Ampicillin	H_2O	100	50	100	5	50
Chloramphenicol	EtOH 100%	30	20	30	1	2
Gentamycin	H_2O	50	15	20	0,5	5
Kanamycin	H_2O	50	30	50	5	20
Nalidixic Acid	NaOH 0,2M	20	15	30	15	20
Oxytetracyclin	EtOH 50%	12,5	12,5	12,5	2,5	5
Rifampicin	MeOH 100%	50	25	50	2,5	5
Spectinomycin	H_2O	100	100	100	25	50
Streptomycin	H_2O	100	50	100	5	5
Glucose	H_2O	20%	-	-	0,20%	0,20%
Sucrose	H_2O	30%	-	-	0,30%	0,30%
Xylose	H_2O	10%	-	-	0,10%	0,10%
Vanillate	H_2O	50mM	-	-	0,5mM	0,5mM

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